Multistage and multiple biomass approaches to efficient biological nitrogen removal using biofilm cultures

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I hereby declare that this thesis is my own account of my research and contains as its main content work that has not previously been submitted for a degree at any university.

Leonie Hughes

Nitrogen is nitrogen, it passes miraculously from the air into plants, from these into animals, and from animals to us; when its function in our body is exhausted, we eliminate it, but it still remains nitrogen, aseptic, innocent.

Primo Levi in *The Periodic Table*

To Dean, thankyou

ABSTRACT

Nitrogen removal from wastewater is important for the prevention of significant health and environmental impacts such as eutrophication. Nitrogen removal is achieved by the combined action of nitrification and denitrification. Nitrification is performed by autotrophic, slow growing microorganisms that require oxygen and are inhibited in the presence of denitrifiers when oxygen and COD are available due to competition for oxygen. Denitrification however, performed by relatively fast growing heterotrophic bacteria, is inhibited by oxygen and requires COD. This implies that nitrification and denitrification are mutually exclusive. The supply of oxygen to a fresh wastewater, high in ammonia and COD, causes waste of both oxygen and COD. Conservation of COD is therefore critical to efficient wastewater treatment. The approach investigated in this study to achieve complete nitrogen removal was to physically separate the nitrification and denitrification biomasses into separate bioreactors, supplying each with appropriate conditions for growth and activity.

A storage driven denitrification sequencing batch biofilm reactor (SDDR) was established which exhibited a high level of COD storage (up to 80% of influent COD) as poly-β-hydroxybutyrate capable of removing >99% of nitrogen from wastewaters with a C/N ratio of 4.7 kg COD/kg $N–NO_3^-$. The SDDR was combined in sequential operation with a nitrification reactor to achieve complete nitrogen removal. The multiple stage, multiple biomass reactor was operated in sequence, with Phase 1 - COD storage in the storage driven denitrification biofilm; Phase 2 - ammonia oxidation in the nitrification reactor; and Phase 3 - nitrate reduction using the stored COD in the storage driven denitrification reactor. The overall rate of nitrogen removal observed was up to

1.1 mmole NH_3 L^{-1} h^{-1} and >99% of nitrogen could be removed from wastewaters with a low C/N ratio of 3.9 kg COD/kg $N–NH_3$.

The multiple stage, multiple biomass system was limited in overall nitrogen removal the reduction in pH caused by nitrification. A parallel nitrification-denitrification (PND) reactor was developed in response to the pH control issue. The PND reactor was operated with Phase 1 – COD storage in the storage driven denitrification biofilm and Phase 2 – simultaneous circulation of reactor liquor between the denitrification and nitrification biofilms to achieve complete nitrogen removal and transfer of protons. The PND reactor performed competitively with the multistage reactor (removal of >99% nitrogen from wastewaters with feed ratios of 3.4 kg COD/kg $N–NH_3$) without the need for addition of buffering material to moderate the pH.

Contents

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Abbreviations

Anammox	Anaerobic AMMonium Oxidation
AOB	Ammonia (ammonium) oxidising bacteria
APSBBR	Alternating pumped sequencing batch biofilm reactor
AUP	Acetate uptake phase
BNR	Biological nitrogen removal
BOD	Biochemical oxygen demand
c_L	DO concentration
C/N feed	Ratio of carbon (expressed as kg COD) to nitrogen (expressed as kg N) in the feed
C/N ratio of removal	Ratio of amount of carbon consumed (expressed as kg COD) to amount of nitrogen consumed (expressed as kg N) in a trial
COD	Chemical oxygen demand (see Appendix A for derivation). In this thesis, COD is mainly used to reflect the biodegradable COD in the feed
CANON	Completely autotrophic nitrogen removal over nitrite
c_S	Saturation DO concentration
DNP	Denitrification phase
DO	Dissolved oxygen
EBPR	Enhanced biological phosphate removal
EPS	Extracellular polymeric substances
ETC	Electron transport chain
FISH	Fluorescence *in situ* hybridisation
GAO	Glycogen accumulating organisms
GC	Gas chromatography
k_La	Oxygen mass transfer coefficient (h^{-1})
K_m	Michaelis-Menten constant
NOB	Nitrite oxidising bacteria
NO_x	Collective nitrogen oxide species
ORP	Oxidation reduction potential
OUR	Oxygen uptake rate
PAO	Poly phosphate accumulating organisms

PHA	Poly hydroxyalkanoate
PHB	Poly-β-hydroxybutyrate
pK_a	Negative logarithm (base 10) of the acid dissociation constant
PND	Parallel nitrification-denitrification
SBR	Sequencing batch reactor
SND	Simultaneous nitrification-denitrification
SOUR	Specific oxygen uptake rate
TCA	Tricarboxylic acid cycle
TF	Trickling filter
TKN	Total Kjeldahl nitrogen
TS	Total solids
VFA	Volatile fatty acids
V_{max}	Maximum biochemical reaction velocity
VS	Volatile solids
WWTP	Wastewater treatment plant

Chapter 1. Introduction

1.1 The impact of nitrogenous waste on the environment

Nitrogen availability and demand is intimately tied to the sustainability of life on earth. While nitrogen is an absolute necessity for the growth of organisms, high levels in aquatic environments can disturb the nutrient balance sufficiently to cause dramatic toxic effects on fish [1]. In contrast, insufficient nitrogen in soils can considerably limit plant growth and lead to soil erosion in the long term due to lack of canopy protection of the soil surface from rain, wind and animal activity [1, 2].

The global environmental nitrogen cycle involves both naturally occurring chemical and biological reactions and industrial processes (Figure 1.1). Nitrogen gas is removed from the atmosphere for conversion into biomass (microbes, plants, animals) and returned to the atmosphere when the biomass is degraded. Fixation of nitrogen gas from the atmosphere can proceed biologically or industrially, by conversion into ammonia. Ammonia has a number of fates once it has been fixed. It can be converted into biomass through assimilation and subsequently released through degradation. It can also be converted to nitrate by nitrification while denitrification releases the nitrogen back into the atmosphere as nitrogen gas. The biologically mediated reactions nitrification and denitrification, form key components of nitrogen removal from wastewater.

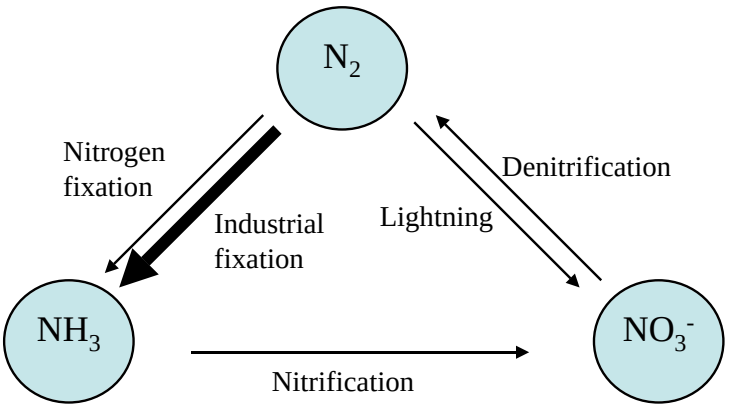

Figure 1.1 A brief summary of the principle chemical and biochemical processes involved in the environmental nitrogen cycle. Biological nitrogen removal from wastewater utilises the naturally occurring pathways of nitrification and denitrification.

Ammonia is the principle form of nitrogen in municipal wastewaters [3], making up approximately 60% of the nitrogen observed [4]. Organically bound nitrogen from proteins and amino acids makes up the remainder [4]. The organic sources of nitrogen yield ammonia when broken down by degradative processes. Due to a lack of oxidation in the wastewater stream, there is usually only a small amount, typically less than 1% of total nitrogen present, as nitrite and nitrate observed [4].

Ammonia has a substantial impact on the environment [1, 2, 5]. The toxicity of ammonia to aquatic organisms is observed at concentrations as low as 0.03 mM [4]. An increase in pH of a water body increases the toxicity of ammonia, presumably as a result of the increased ratio of ammonia to ammonium (Figure 1.2).

It was thought that nitrate, when contaminating drinking water supplies, accumulated in biological systems, blocking the transport to the blood of oxygen by haemoglobin in young children (blue baby syndrome) [4], although substantial epidemiological studies have not found consistent evidence to support this [6]. Abundance of nitrogen in surface

waters stimulates the growth of algae and aquatic plants [1, 6, 7]. When the nutrient balance is sufficiently disturbed, algae can proliferate, causing blooms [5].

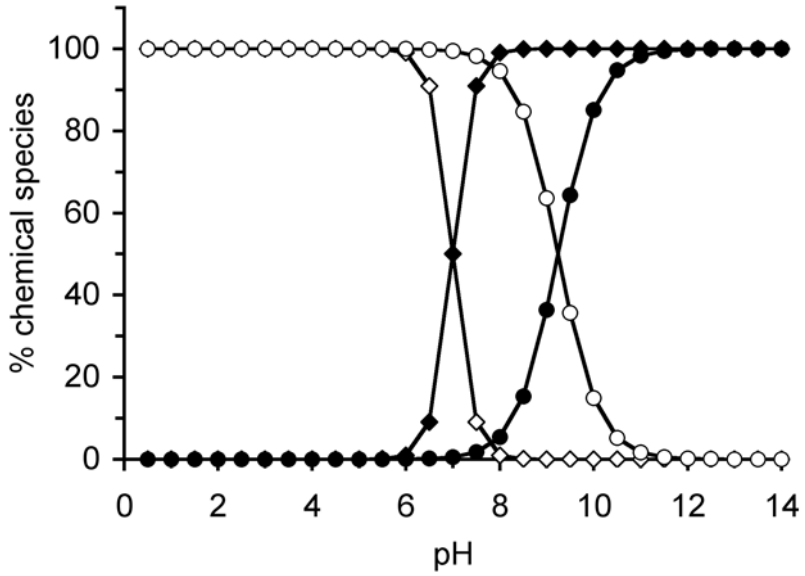

Figure 1.2 The effect of pH on the chemical speciation of the ammonia-ammonium conjugate acid-base pair. Proton-hydroxide speciation shown for contrast: Protons (◇), hydroxide ions (◆), ammonium ions (○) and ammonia (●).

The contribution to oxygen demand by nitrification can be as high as that for organic carbon in raw wastewater streams [4]. The resulting oxygen demand caused by biomass growth diminishes the availability of oxygen for other marine and freshwater organisms, which may lead to large fish kills [1].

The removal of nitrogen from municipal, and other, wastewater streams prior to disposal is of vital environmental importance. This is especially true as populations increase and society becomes more urbanised, contributing increased anthropogenic sources of nitrogen pollution [1, 2]. In addition, legislation concerning effluent quality in terms of nutrient release are becoming stricter with better understanding of environmental impacts [8].

Nitrogen must be treated in a way that best attains a compromise between environmental impact and cost of treatment. The exploitation of the processes of nitrification and denitrification, important components of the environmental nitrogen cycle, is a key approach to nutrient removal from wastewater streams. These processes have been used in advanced wastewater treatment for many decades. Each process is distinct and has its own limitations that need to be understood for successful wastewater treatment.

1.2 Nitrogen removal microbiology and biochemistry

The need for nitrogen removal from wastewater is clear. It is usually performed by the concurrent or consecutive action of nitrifiers and denitrifiers, the biochemistry of which need to be clearly understood for increased insight of the issues in removing nitrogen from wastewater sources and to improve performance of nitrogen removal systems.

Nitrification and denitrification link nitrogen *via* biological processing from the lowest oxidation state (ammonia) to the highest oxidation state (nitrate) and back to its elemental state (nitrogen gas) (Figure 1.3).

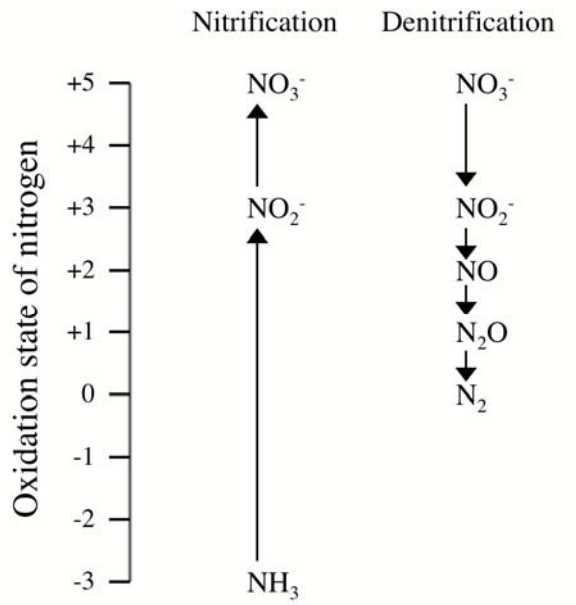

Figure 1.3 Change in oxidation state of nitrogen during nitrification and denitrification. There is redundancy and energy wastage in the process demonstrated by the common intermediate, nitrite.

1.2.1 Nitrification

Nitrification, the sequential oxidation of ammonia to nitrite and then to nitrate, is typically performed by chemolithoautotrophic bacteria [3]. The energy yielded from nitrification is so low that only a specialist niche of organisms exploit the process [3]. The energy that is obtained from the oxidation reactions is nearly entirely spent on the generation of reducing power within the cell required for the fixation of carbon dioxide. Some estimates have shown up to 80% of the energy yield from nitrogen oxidation being used to reduce NAD^+ and subsequently drive carbon dioxide fixation in the Calvin cycle [3, 9].

Inorganic carbon is required by these organisms from which they can synthesise all complex chemicals required for growth and maintenance [3]. With few exceptions, the nitrifying bacteria are obligately dependent on carbon dioxide [9]. Those nitrite

oxidisers that are able to metabolise more complex organic carbon have been observed to grow even slower on heterotrophic energy sources than on nitrite as the energy source [3, 9]. When nitrification was initially observed it was thought that attachment to a solid matrix was a requirement for growth as nitrifiers seemed difficult to grow in suspension [3]. This has since been overcome. There is a clear tendency, however, for them to grow as a biofilm if a surface is available for attachment.

Given the low energy that is generated from nitrification, it is no surprise that the organisms that perform these reactions are very slow growing compared to heterotrophic species [3, 5, 10]. Maintenance of a sufficient population of the relatively slow growing nitrifiers in wastewater is managed by manipulation of solids retention time and sludge production. The growth rate of nitrifiers is subject to control by temperature, pH, oxygen tension and substrate concentration [11].

Enzymology of nitrification

Two reactions performed by two separate gram negative bacteria make up the process of nitrification: the oxidation of ammonia to nitrite, known as nitritification performed by *Nitrosomonas* and *Nitrosococcus* species, and the subsequent oxidation of nitrite to nitrate, known as nitratification and performed by *Nitrobacter* [3]. These organisms, as well as a number of less abundant others, form the family Nitrobacteraceae. This family classification is based solely on physiological properties and thus the many different examples of life based on the low energy yield from ammonia oxidation have not necessarily evolved in common [3]. Nitritification is shown below:

$$NH_3 + 1.5\ O_2 \rightarrow NO_2^- + H^+ + H_2O \qquad \text{Equation 1.1}$$

The production of nitrite from ammonia proceeds *via* the intermediate hydroxylamine [9]. Hydroxylamine has been observed to be a sufficient substrate for nitritification regardless of whether ammonia is also present. Nitritification requires a reducing agent and hydroxylamine can serve this purpose if present [9]. Ammonia monoxygenase, the enzyme catalysing the production of hydroxylamine, takes electrons from the electron transport chain (ETC) while hydroxylamine oxidoreductase feeds them in [9]. Both reactions are reversible and thought to interact with the underlying ETC around the ubiquinone-cytochrome *b* region [9]. The monoxygenase enzyme can also oxidise methane, methanol and carbon monoxide [9]. While some methane oxidising organisms possess a similar monoxygenase, none have been shown to be capable of surviving solely on ammonia [9]. The nitritification reactions occur in the periplasm and the cytoplasmic membrane, limiting cytoplasmic exposure to toxic nitrite and may link the reactions close to proton motive force generation sites [9].

Under conditions with minimal oxygen availability, the oxidation of ammonia by nitritifiers, or ammonia oxidising bacteria (AOB), produces nitrous oxide and nitric oxide [9]. A nitrite reductase has been found which can reduce either nitrite, to the gaseous nitrogen oxides, or oxygen. The generation of nitric and nitrous oxide, *via* this process, may be a conservation mechanism of oxygen for the cell under limiting oxygen supply [9]. This enzyme has no ability to extract protons from the cytoplasm however, and therefore cannot play a role in balancing the redox potential of the cell [9].

It is ammonia, rather than ammonium that is the true substrate of nitritification. The apparent Michaelis-Menten constant of the reaction (K_m), with respect to total ammonia concentration, changes with pH while the K_m for free ammonia is independent of pH [9]. The usual operational pH of a wastewater treatment plant [12] however, causes the

dominant form of ammonia to be ammonium (Figure 1.2). Thus, Equation 1.1 should be modified accordingly:

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2\ H^+ + H_2O \qquad \text{Equation 1.2}$$

It can be seen that the yield of protons is twice as much at lower pHs (less than the pK_a of ammonium; Equation 1.2 *vs* 1.1) where ammonium must be stripped of a proton in order to facilitate nitritification.

Nitratification appears to proceed without any intermediates *via* the action of the membrane bound enzyme nitrite oxidoreductase. Nitratifiers, or nitrite oxidising bacteria (NOB), perform a reversible reaction that intersects with the ETC at the cytochrome aa_3 level [9] and is shown below.

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \qquad \text{Equation 1.3}$$

The influence of environmental conditions on nitrification

Nitritification and nitratification are inhibited by high concentrations of both their substrates [7, 9, 10] and products [7, 9, 10]. Molecular oxygen is an absolute requirement for growth in nitrifying cultures [9]. The presence of COD has been shown to inhibit nitrification activity in true autotrophs [9] and is most likely the result of competition for oxygen between nitrifiers and heterotrophs. The production of protons in the first reaction in nitrification is clear (Equations 1.1 and 1.2). The resulting reduction in pH can severely inhibit the activity of nitrifiers [9]. There is some thought that the growth of nitrifiers in biofilms rather than suspended culture may afford some

protection from low pH environments. This is not supported by literature evidence however where extreme localised pH depressions have been observed in bacteria immobilised in a matrix [13]. In fact, it has been observed that within biofilms performing nitrification, the reduction in pH of the bulk liquid as a result of nitrification activity can be much less than that which may be occurring within the biofilm matrix [13].

Despite the low growth rate and various inhibitors to competitiveness that the nitrifiers face, they are found in a wide range of environments, particularly where organic material is being mineralised to release nutrients such as in soils, composts and sewage [11]. Key considerations in the management of nitrification for wastewater treatment include the slow growth of nitrifiers, inhibition by low pH and tendency to grow in biofilms.

1.2.2 Denitrification

Nitrate reduction is performed by a range of bacterial species for a variety of outcomes. Nitrate can be taken up by cells and assimilatively reduced to ammonia, to produce biomass, a reaction that is inhibited by the presence of ammonia [14, 15]. Dissimilatory ammonia production, or ammonification, is also a possible fate of nitrate under reducing conditions. Ammonification is thought to function as either an electron sink for bacteria or to remove toxic compounds from the cell [14, 15]. In addition to these is the dissimilatory pathway, where nitrate is reduced to nitrogen gas for the purposes of respiration under anaerobic, or more correctly anoxic, conditions [14, 15]. This is the principle mechanism through which nitrogen is lost from fixed sources [16].

Nitrate can be used as an alternative to oxygen as the terminal electron acceptor in respiration [14]. The similarity of the denitrification pathway with that of aerobic respiration is demonstrated by the similar structural properties of the terminal oxidases of aerobic respiration to those in anoxic respiration [14]. Organisms usually possess the capability both of the dissimilatory nitrate reduction pathways, that is denitrification and ammonification, but only one of these may be expressed at any time [14]. Those organisms capable of denitrification are ubiquitous in the environment. They are found in all environments including Antarctic and arctic soils [11].

Successive reactions of denitrification

Denitrification is the complete reduction of nitrate to elemental nitrogen gas by bacterial action. Four separate reactions occur to achieve the complete process, which can be performed by a single organism or a group of cooperative microorganisms [14, 17]. The four reactions can be thought of as modular in action as each can be, to varying degrees, independently regulated [14]. Complete denitrification occurs only when the four reactions are activated, expressed and function concomitantly [14]. Convention defines denitrification only as the respiration of nitrogen oxides to produce a gaseous end product [14, 16]. The redox equations for the four reactions involved in denitrification are shown below (Equations 1.4 to 1.7), adjusted to show the dominant chemical species at the normal operational pH of wastewater treatment plants:

$$2\ CH_3COO^- + 8\ NO_3^- \rightarrow 4\ HCO_3^- + 8\ NO_2^- + 2\ H^+ \qquad \text{Equation 1.4}$$

$$CH_3COO^- + 8\ NO_2^- + 7\ H^+ \rightarrow 2\ HCO_3^- + 8\ NO + 5\ H_2O \qquad \text{Equation 1.5}$$

$$CH_3COO^- + 8\ NO \rightarrow 2\ HCO_3^- + 4\ N_2O + H^+ \qquad \text{Equation 1.6}$$

$$CH_3COO^- + 4\ N_2O \rightarrow 2\ HCO_3^- + 4\ N_2 + H^+ \qquad \text{Equation 1.7}$$

$$5\ CH_3COO^- + 8\ NO_3^- + 3\ H^+ \rightarrow 10\ HCO_3^- + 4\ N_2 + 4\ H_2O \qquad \text{Equation 1.8}$$

It is clear that a source of electrons is required for the reactions above to proceed. The contribution to this native chemical oxygen demand (COD) by volatile fatty acids, such as acetate, mean that these chemical species are particularly important to denitrification of wastewater [12]. In wastewater treatment plants, the native COD contained in the wastewater stream often needs to be supplemented with methanol [4].

The minimum chemical requirement of acetate for the complete reduction of nitrate is five moles of acetate per eight moles of nitrate (Equation 1.8), or 2.86 kg COD/kg N-NO_3^- (Appendix A). In practice, this ratio is insufficient for bacteria to perform complete denitrification as the energy yielded from these compounds is also required for cell growth and maintenance processes. In addition, the efficiency of ATP generation *via* nitrate respiration is lower than that from oxygen [4]. A ratio of 4 kg COD/kg N-NO3- is a more appropriate reflection of metabolic requirements [18] but may be even higher [19]. The supply of COD, or more importantly biologically available COD, in raw wastewater streams varies greatly but is typically insufficient to allow complete denitrification as it is not generally well conserved [20]. Some particularly recalcitrant wastewaters, such as piggery waste, are considerably higher in nutrients with relatively low COD available for nitrogen removal [21] increasing the challenge for successful biological nitrogen removal (BNR).

Enzymology and respiration of nitrogen oxide reduction

Respiration as a result of nitrate, nitrite and nitrous oxide reduction is life supporting [14]. The radical nitric oxide is not known to allow respiration alone without coupling to either nitrite or nitrous oxide reduction. The genes for enzymes in bacterial reduction or respiration of nitrite and nitric oxide are co-located in many denitrifiers and are interdependently controlled at the transcription and enzyme activity levels [14].

While the membrane bound nitrate reductase has its catalytic site facing the cytoplasm, the catalytic site for nitrite reductase is in the periplasmic space [14, 17]. This implies that an active transport mechanism is required for the movement of both nitrite and nitrate to the sites where they are required. This may seem a costly process but it allows the cell to remain impervious to the production and presence of the radical nitric oxide, a known bactericide [14]. When denitrification occurs under steady state conditions, extracellular nitric oxide is observed at low nanomolar concentrations [14]. Thus the chemical species of interest, both for their ability to act as a source of respiratory power and their relative observable abundance, are nitrate, nitrite and nitrous oxide.

Denitrification is performed by facultative heterotrophic organisms, those that will preferentially use oxygen if available but have the ability to grow under anoxic conditions because they can substitute a nitrogen oxide species for oxygen and continue to respire [14]. As shown above (Equations 1.4 to 1.8), a source of electrons is required to allow each of the reactions in denitrification. The energy released from nitrate reduction is less than that from oxygen and oxygen is therefore the preferred terminal electron acceptor [5, 14].

Regulation of denitrification – a complex process

The expression of the denitrification pathway is very sensitive to oxygen, although a low oxygen tension is alone not enough to induce expression of the denitrification enzymes required [14, 17]. The presence of a respirable nitrogen oxide species (see above) is also required [14]. To a less clear extent, the presence of particular metal ions, such as molybdenum and iron, is also important as these form core components of the active sites of the functional enzymes [14]. The gene clusters for these pathways have multiple promoter sites that allow subtle control of expression [14]. It is clearly too simplistic to conclude that the regulation is based entirely on the absence of oxygen and the presence of nitrate.

Two forms of nitrate reductase exist in gram negative organisms. One is membrane bound as discussed above, but an additional enzyme, acting in an identical chemical reaction is found in the periplasm [14]. The periplasmic enzyme is thought to reduce the stress of transition from oxic to anoxic conditions as it is not inhibited by oxygen [14]. The genes for these two differing nitrate reductase enzymes are not co-located on adjacent loci [14].

The metabolic and environmental conditions amenable to performing denitrification differ markedly from those required for nitrification. A major challenge for wastewater treatment practitioners is the management of each process for complete nitrogen removal with efficient use of oxygen and COD.

1.2.3 Are denitrification and nitrification mutually exclusive processes?

Activated sludge, the biosolid that underpins biological wastewater treatment, consists of a mixed, naturally occurring bacterial culture that includes nitrifiers, denitrifiers and other heterotrophs as well as higher organisms such as nematodes [22, 23]. As the principle form of nitrogen entering a municipal, domestic wastewater treatment plant is ammonia as opposed to nitrite or nitrate (Section 1.1), nitrification should logically occur before denitrification. COD is abundantly available in the influent stream to a plant. However, nitrifiers are inhibited by COD due to competition for oxygen in the presence of heterotrophs. In addition, nitrifiers do not compete well for oxygen in the presence of heterotrophs. The aeration of the wastewater and sludge during the first stages of wastewater treatment would therefore cause both the wastage of oxygen, by inadvertent supply to heterotrophs, and of COD, which the heterotrophs consume when oxygen is abundant.

The wasting of COD requires that strategies be developed to supplement the supply or reduce the waste of it. In many wastewater treatment plants, the approach is to add methanol as a readily degradable external carbon source [4], increasing the cost of operating the plant. An alternative is to introduce raw wastewater into the denitrification stage. This has the disadvantage of potentially causing untreated ammonia release into the environment [24].

Oxygen inhibits denitrification and therefore it is important to strictly limit its supply during this phase of wastewater treatment. Autotrophic nitrifiers also grow considerably slower than the facultative heterotrophs performing denitrification. As a result, in wastewater treatment, a high solids retention time is required to ensure that a substantial

population, and hence activity level, of nitrifiers is maintained [4]. This can be achieved by ensuring that the biomass washout rate is lower than the nitrifier growth rate [4]. A consequence of having a low biomass washout rate or high solids retention time is that the much faster growing heterotrophs can subsequently cause sludge bulking [25], a major issue in the management of activated sludge processes, increasing the need for sludge treatment.

The separate processes of nitrification and denitrification that are needed for complete nitrogen removal in wastewater treatment appear to be mutually exclusive when their differing requirements for oxygen and COD are considered as well as their respective growth rates. It is essential, however, that both processes occur efficiently and economically to reduce costs and environmental impact of nitrogen. A number of strategies have been attempted to address these issues.

1.3 Storage and conservation of COD

Technological developments in biological nitrogen removal from wastewater have led to an increase in transient substrate supply conditions in activated sludge systems, for example oxygen variation [26, 27]. The sequencing batch reactor (SBR) is one example of this change. While bacteria have long been known to store excess substrate when experiencing nutrient limitation, the exploitation of storage is a relatively new direction in wastewater treatment. It seems logical that the conservation of native COD in the form of intracellular storage would be a valuable tool in the efficient removal of nitrogen from wastewaters.

Many bacteria are able to internally accumulate compounds for carbon and energy storage. For a compound to be a true energy store it must meet three criteria: 1) It must be accumulated intracellularly when the supply of exogenous substrate is in excess of requirements, usually dictated by limitation of some other nutrient such as nitrogen or oxygen; 2) It must be degraded when the supply of exogenous substrate has been exhausted; and 3) It must be degraded in such a way that it allows the cell to produce energy for growth and facilitates the cells advantage over other species [28].

A key criterion in classifying an energy reserve chemical for a microbe is the intracellular accumulation. This particular statement allows easy differentiation from other polymeric material that may be produced, for example, as a method of removing toxic metabolic end products, which are often stored extracellularly. There are three main types of storage compounds observed in bacteria [28]. These are glycogen, polyphosphate and lipids such as poly hydroxyalkanoates (PHA), for example poly-β-hydroxybutryic acid (PHB). All three have important consequences in wastewater treatment. PHB is a valuable source of COD in wastewater treatment. It is useful, as a source of COD, in both nitrogen removal *via* denitrification, and in phosphorus removal *via* polyphosphate production [26, 29, 30]. Glycogen is also useful as an electron source for denitrification but is often the cause of a breakdown in enhanced biological phosphorus removal (EBPR) due to competition between glycogen accumulating organisms (GAO) and poly phosphate accumulating organisms (PAO) [31, 32] and thus may only be useful where nitrogen removal alone is required. Where both nitrogen and phosphate removal is required, careful control of population dynamics is required to maintain PAOs such as in the simultaneous nitrification, denitrification and phosphorus removal (SNDPR) [33] and Dephanox [34] systems.

PHB is very important for nitrogen removal as it is predominantly formed by the accumulation of volatile fatty acids (VFA) such as acetate [27], which are an important form of soluble COD in typical municipal wastewaters. The metabolism of VFAs is well understood. Influent COD such as acetate is channel into energy production *via* the tricarboxylic acid (TCA) cycle or PHB production are linked through acetyl CoA (Figure 1.4). When growth conditions are balanced, that is both electron donor and acceptor supply are abundant, there is a large internal concentration of CoA and a low ratio of NADH/NAD. These conditions inhibit the conversion of acetyl CoA into acetoacetyl CoA by α ketothiolase [35], the first step in PHB production (Figure 1.4*A*). When oxygen, or nitrate, is low while carbon supply is relatively high, there is a low amount of CoA and high ratio of NADH/NAD [35]. As citrate synthase and isocitrate dehydrogenase, enzymes involved in the TCA cycle, are inhibited by high NADH, the low electron acceptor supply causes the TCA cycle to be depressed and activates the PHB production cycle [35] (Figure 1.4*B*). Some bacteria have been observed to have constitutively expressed enzymes in the PHB production pathway [35].

PHB has been shown to be a suitable electron donor for the reduction of nitrogen although it is generally hydrolysed slower than an exogenous energy source, such as acetate, can be oxidised [18, 26]. Therefore denitrification usually proceeds at a lower rate with PHB than with acetate. In turn, the degradation of stored PHB is lower under anoxic conditions than under aerobic conditions [18, 26], presumably due to the lower efficiency of ATP production from nitrate as a terminal electron acceptor compared to oxygen. While this may appear to be a disadvantage of PHB, or other storage polymers, as an electron donor for denitrification, it does potentially allow for conservation of COD specifically for use in denitrification rather than wasted under aerobic conditions.

Cells can accumulate large amounts of PHB even up to 86% of the cell dry weight [28]. The maximum theoretical amount that may be produced due to physical restrictions of cells by *Alcaligens eutrophus* was found to be 80% dry cell mass [36]. In practice, this level of storage is unlikely to be observed in routine wastewater treatment due to limited COD supply. PHB production can be manipulated by the control of electron acceptor supply (*eg* oxygen) to encourage storage. Third *et al* [37] have confirmed that at least 17% of exogenous acetate must be oxidised to provide sufficient energy for the uptake and activation of the remainder acetate for PHB production. Over a range of DO set points monitored, the maximum yield of PHB was observed at 0 mg L^{-1} (oxygen mass transport coefficient (k_La) = 12 h^{-1}) with 0.68 Cmol of PHB produced per Cmol of acetate. At higher DO set points, or higher k_La values, an increasing amount of acetate was oxidised, as would be expected from the regulation of PHB production. At lower k_La values, the supply of oxygen was insufficient for complete uptake of acetate, reducing the production of PHB.

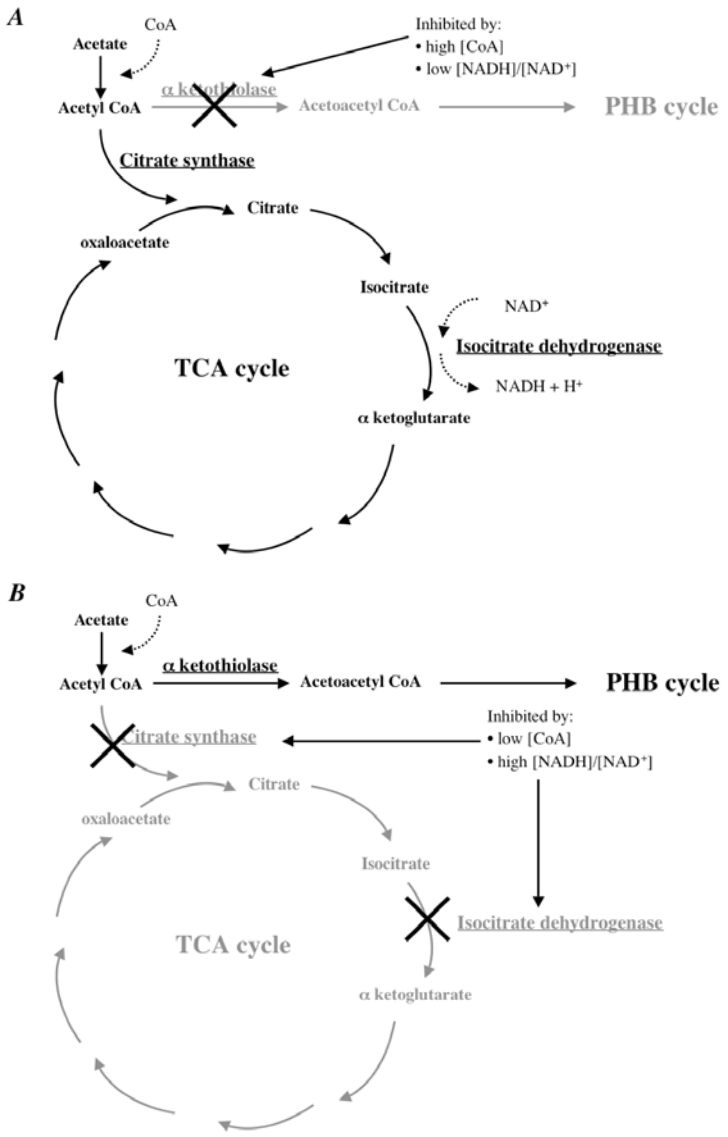

Figure 1.4 Simplified schematic of the regulation of PHB production under balanced growth conditions (*A*: abundant electron acceptor and donor available) and unbalanced growth conditions (*B*: insufficient electron acceptor supply for oxidation of donor supplied). Under balanced growth conditions (*A*), there is a concentration of CoA and a low ratio of NADH/NAD in the cell that inhibits α ketothiolase and so production of PHB is downregulated. Under balanced growth conditions (*B*), there is a low concentration of CoA and a high ratio of NADH/NAD in the cell that inhibits citrate synthase and isocitrate dehydrogenase and so the TCA cycle is downregulated. Enzymes are underlined, substrates are shown in bold and cofactors are shown in normal text.

1.4 Wastewater treatment approaches for complete BNR

1.4.1 Traditional wastewater treatment

The two key reactions in the removal of nitrogen from wastewater, nitrification and denitrification, appear to have mutually exclusive requirements for optimised performance (Section 1.2.3). The combined action of nitrification and denitrification in traditional wastewater treatment must be achieved continuously as wastewater is fed into the process continuously. The different conditions for nitrification and denitrification are supplied by plug flow operation in which there is a gradient along the axis of flow (Figure 1.5*A*) [38]. A single microbial population is maintained through the entire process. Nitrifiers are active initially where there is plentiful oxygen supply while denitrifier activity dominates where oxygen has been exhausted. Under plug flow type operation, the supply of oxygen in the initial stage of wastewater treatment allows heterotrophic organisms to use oxygen for the oxidation of COD in the waste stream. The consumption of oxygen and COD by the heterotrophic organisms present causes the waste of both of these critical substrates. The supply of COD can be supplemented by the addition of an external COD source such as methanol to the plant where denitrification is occurring. The use of such a substrate increases the cost of operation.

Plug flow operation can also be implemented in reverse order as a means of supplying native COD for denitrification. So called predenitrification [40] systems (Figure 1.5*B*) lower the hydraulic capacity of the plant due to the need to mix the wastewater inflow with some recirculated partially treated wastewater.

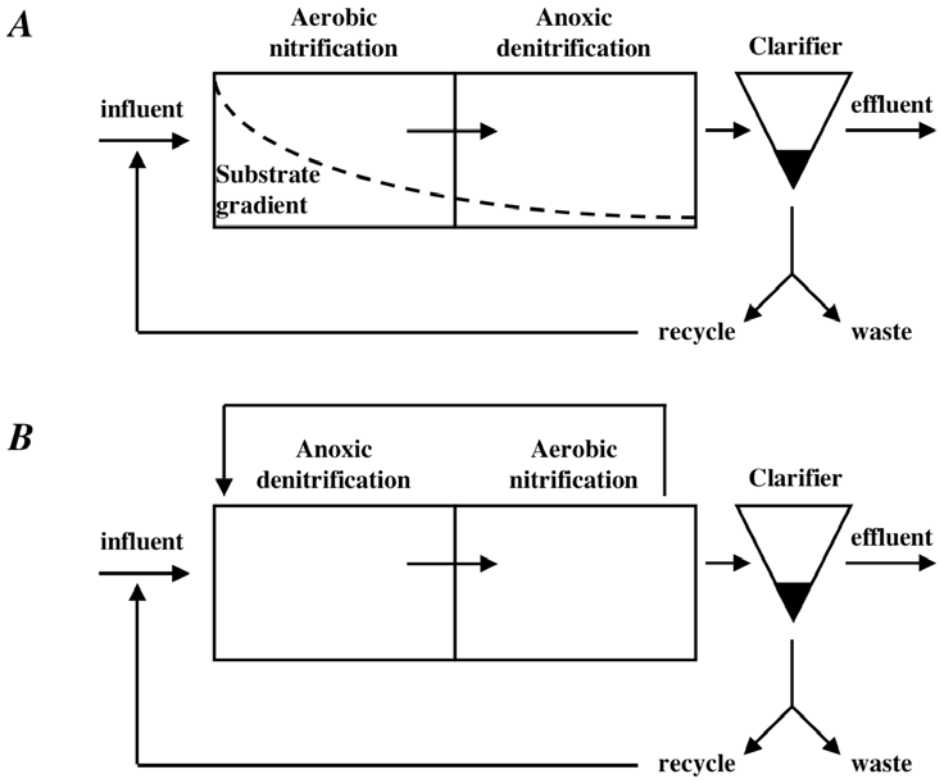

Figure 1.5 *A*: A simplified diagram of the conventional design on a plug flow wastewater treatment plant for the removal of nitrogen and carbon, showing the decreasing substrate concentration over the length of the reactor (Figure from [39]). *B*: A simplified diagram of a predenitrification plug flow wastewater treatment plant design aimed at efficiently using the COD available in the wastewater for denitrification (Figure from [39]).

1.4.2 SND for nitrogen removal

An alternative approach to traditional wastewater treatment processes, is simultaneous nitrification denitrification (SND). SND usually involves a single biomass, which is maintained at a particular dissolved oxygen concentration chosen in order to reach a compromise between rate of nitrification and inhibition of denitrification. Under such an arrangement, neither nitrification nor denitrification occurs at maximum rates (Figure 1.6).

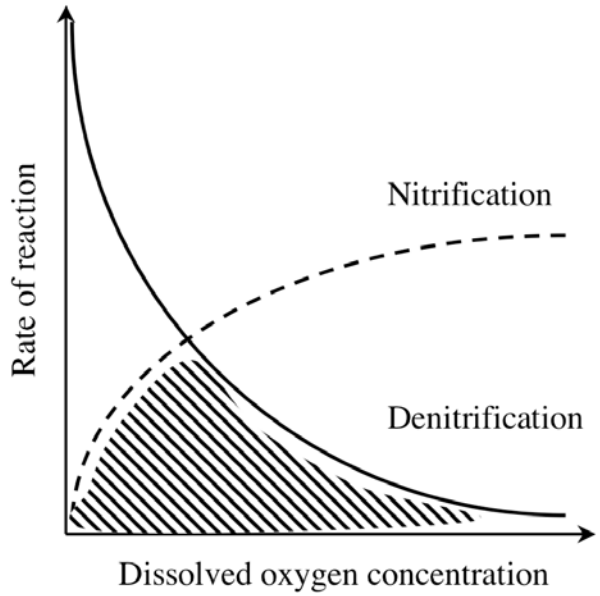

Figure 1.6 The effect of dissolved oxygen concentration on the rates of nitrification (dashed line) and denitrification (solid line). The shaded area indicates the notional operational target of SND.

A key advantage of the single sludge approach found in SND reactors is the possible reduction in required COD supply based on the short cut between nitrification and denitrification *via* nitrite (Figure 1.7), a process facilitated by high ammonia concentrations [41]. Under conditions favouring SND (DO 0.5 mg L^{-1}), fast reduction of nitrite was observed only as long as ammonia was present in the medium [41]. There are a number of explanations that may cause this effect: competition between AOBs and NOBs for oxygen; ammonia being a source of electrons for nitrite reduction or NOB inhibition by ammonia [41].

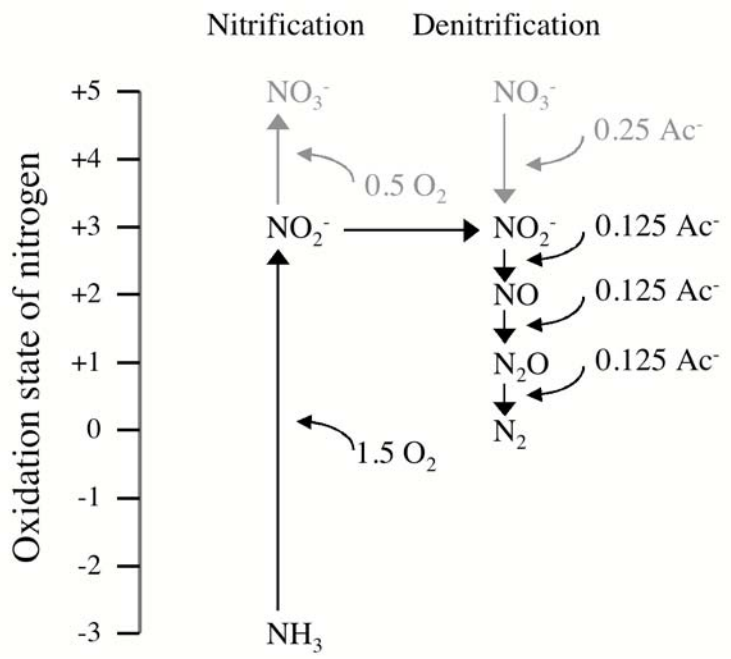

Figure 1.7 Theoretical short circuit between nitrification and denitrification providing savings of 40% COD (0.25 mol acetate/0.625 mol acetate) and 25% oxygen (0.5 mol oxygen/2 mol oxygen) as a result of nitrification-denitrification *via* nitrite.

The exact mechanism of SND in mixed cultures is not clear [41]. Some authors suggest that the internal structure of flocs in suspended cultures play an important role [29, 41-43]. Where a highly organised floc structure is observed (or hypothesised), it is possible that the consumption of oxygen by an outer layer of nitrification organisms creates a gradient within the floc leading to the creation of an internal anoxic space for denitrifiers to flourish in. Alternatively, the possibility of denitrification occurring under high oxygen supply in some microbes may explain the situation in some treatment plants [15, 44-47].

1.4.3 Wastewater treatment with physically separated nitrifying and denitrifying biomasses

Traditional and SND approaches to nitrogen removal each have a number of drawbacks. For traditional plants, the loss of COD and oxygen represents a significant cost and efficiency burden. Under SND operation, neither nitrification nor denitrification can proceed at a maximal rate. Since nitrification and denitrification seem to be mutually exclusive with respect to optimal environmental conditions, it seems reasonable to investigate an alternative approach to wastewater treatment in which the nitrifying and denitrifying biomasses are physically separated.

The specific retention of separate populations of bacteria in multibiomass systems is achieved and maintained by the control of different environmental and substrate supply conditions. Autotrophic nitrifiers do not grow competitively in the anoxic denitrification zones of multistage reactors due to the lack of oxygen while the heterotrophic organisms do not compete well in the aerobic zone if all COD has been removed [24]. Sludge recycle between the COD storage and denitrification phases (Figure 1.8) has been employed in a number of reactor configurations such as the Dephanox [48] process in which removal of both nitrogen and phosphorus can be achieved. However, the recycle of sludge forward through the process (Figure 1.8) means that some proportion of untreated ammonia will bypass the nitrification stage. Although a small amount of ammonia will be assimilated as biomass, the relative amount of assimilation is low compared to the amount of ammonia available and hence washed out [24, 49].

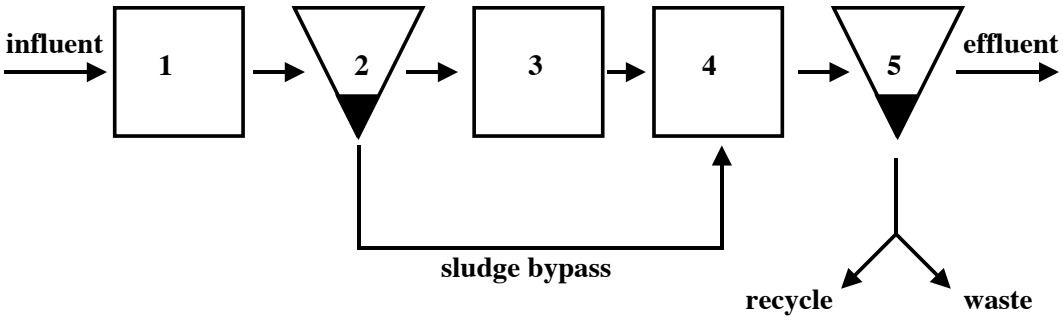

Figure 1.8 Simplified schematic of a multistage and multibiomass wastewater treatment process involving the recycle of sludge (sludge bypass) loaded with stored COD for denitrification. The bypass of sludge past the nitrification stage may cause the release of untreated ammonia. 1: COD sequestration; 2: sludge clarification; 3: nitrification under aerobic conditions; 4: denitrification of nitrified waste using biomass with stored COD from stage 1; 5: clarification of effluent waste stream.

Alternatively, the waste stream can be recirculated while the biomass is retained. For this reason, biofilms are of interest in separated sludge systems. Biofilm based reactors can theoretically increase the biomass 'concentration' when comparing a similar sized suspended culture reactor and hence, may have lower footprint requirements [8]. This is of particular interest with increasingly dense urban populations the volume of wastewater to be treated is high while the land available for treatment is low [8]. Biofilms may also assist in the regulation of population characteristics, for example, the maintenance of a high level of nitrifiers without sludge bulking [8]. As with SND systems, multibiomass reactors may still require supplementary supply of COD when the denitrification phase is reached.

More detailed discussion of other innovative approaches to BNR are included where relevant in each of the experimental chapters that follow.

1.4.4 Suggested reactor design for improved nitrogen removal from wastewater

From the discussion above, it would appear reasonable that a reactor could be designed in which the nitrification and denitrification biomasses were separated in order to provide an optimal environment for each. In addition, since denitrification organisms have been observed to store COD under nutrient limiting conditions (Section 1.3), the initial anoxic supply of wastewater to a denitrification biomass could induce the storage of native COD and thus reduce waste.

The combination of efficient nitrogen removal with intracellular storage of COD could further improve the efficiency of nitrogen removal [29, 41]. In SND systems, this can be achieved by the time dependent control of oxygen for inducing storage conditions followed by nitrogen removal conditions. That is, a limited oxygen supply (eg DO = 0 mg L^{-1}; k_La = 12 h^{-1} [37]) during the feast phase in order to induce unbalanced growth conditions encouraging PHB storage followed by increased oxygen supply conducive for SND to occur.

The time dependent control of oxygen in SND has been successfully applied to achieve good C/N treatment ratios at efficient rates. For example, Third *et al* [29] removed >99% nitrogen at an influent feed ratio of 11 kg COD/kg N-NH_3 and an overall rate (Section 4.2.4) of 0.6 mM h^{-1} with a synthetic wastewater [41]. A local wastewater treatment plant (Woodman Point Wastewater Treatment Plant, Western Australia) has a soluble COD/ammonia content of approximately 11 kg COD/kg N-NH_3 [20]. It is not clear whether all of the soluble COD in the raw wastewater is bioavailable and therefore would exhibit the same performance as Third *et al* [29] observed. Further conservation

of influent COD is required if near complete removal of nitrogen is to be achieved without carbon supplementation.

In a dual biomass system, where the heterotrophic denitrification organisms are separated from the autotrophic nitrifiers, the optimisation of all three stages of nitrogen removal may be achieved. That is, 1) conditions inducing intracellular storage of COD, presumably as PHB by the heterotrophic biomass under limited electron acceptor supply; 2) optimal supply of oxygen to the nitrification biomass for ammonia oxidation of the COD free wastewater from stage 1; and 3) nitrate reduction from the liquor from stage 2 with COD supplied from the sequestered material from stage 1. Maximum COD storage could theoretically be achieved and any excess could be held in reserve for any cycles in which the native COD was insufficient for complete denitrification. The proportion of influent COD that can be stored as PHB under aerobic conditions [50] is higher than that under denitrifying conditions [18] and therefore, an aerobic COD storage phase was chosen.

Very few studies with this approach have been published [40, 51-53]. A major limitation with some of them has been the choice of reactor design for each stage of the wastewater treatment. For example, Jones *et al* [40] employed a suspended culture SBR for the first (COD sequestration) and last (denitrification using the stored substrate) phases. The use of SBR type systems routinely requires the retention of up to 60% of the volume for maintenance of the sludge population [37]. In a multistage system such as this, the result would be the retention of substantial amounts of untreated ammonia from Stage 1 and environmental release after Stage 3. In the BIOFIX process [53], which used biofilms for all stages of nitrogen and COD removal, ammonia was found to adsorb to the biofilm matrix. Thus a biofilm will not completely eliminate the problem

of ammonia release. While the metabolic capacity of the different biomasses was adequate to achieve complete nitrogen removal, the overall removal of nitrogen was limited by the physical retention of untreated ammonia from Stage 1. It would therefore be preferable to use biofilm cultures.

From the knowledge discussed in this review, it is possible to predict some issues with a reactor design of this separated biomass type. Of primary concern, would be the inhibitory reduction in pH due to nitrification. While this thesis investigates the operation of a separated biomass reactor, it also attempts to reduce the impact of such possible negative outcomes due to the physical separation of nitrification and denitrification.

1.5 Scope and outline of this thesis

The research topics presented in this thesis all have the central theme of optimising nitrogen removal in a separated biomass system where the denitrification and nitrification biomasses are physically separated. The philosophy underlying this was to develop an alternative to SND where the key criteria for success were optimised nitrogen conversion in nitrification and denitrification rather than a compromise to achieve both under oxygen limitation. Initial studies were directed at the development of a specialised storage driven denitrification reactor in which alternating feed conditions were applied to alternately supply abundant COD and then abundant electron acceptor (nitrate) (**Chapter 2**). An improved understanding of the factors influencing the storage of COD and associated carbon and energy balance was sought in **Chapter 3**.

The combination of the storage driven denitrification biofilm with a suitable separate nitrification culture was studied in **Chapter 4**. The separation of nitrification from denitrification caused a detrimental reduction in pH and hence an inhibition of nitrification. In addition, the biofilm based denitrification reactor still retained untreated ammonia due to physical adsorption. Due to these two issues encountered in the multistage system, an improvement was sought for achieving complete nitrogen removal analogous to SND using the separated sludge reactors – the parallel nitrification and denitrification (PND) reactor (**Chapter 5**). Further investigations were undertaken to assess the effect of oxygen (**Chapter 6**) and mechanical parameters (**Chapter 7**) on the PND system.

1.6 Thesis objectives

This thesis intends to combine current knowledge about the optimal conditions for nitrification, denitrification and COD storage to develop a wastewater treatment process that minimises oxygen use and COD requirement for maximum nitrogen removal by the action of separated nitrification and denitrification biomass populations. In order to achieve this, the objectives of this thesis were to:

- Ascertain whether a storage driven denitrification biofilm could be developed.
- Determine the level of storage the storage driven denitrification biofilm could achieve.
- Determine if the storage driven denitrification reactor could be combined with a suitable external nitrification reactor for complete nitrogen removal.

- Determine the relative advantages and disadvantages of the separated biomass multistage system relative to the philosophically opposed approach of nitrogen removal in SND.
- Maintaining the separated biomass approach, determine the limitations of multistage nitrogen removal and attempt to solve these using simple operational changes to the multistage reactor to optimise nitrogen removal.
- Understand and optimise the PND reactor in order to achieve relatively high rate of nitrogen removal at relatively low COD/N supply conditions.

Chapter 2. Storage driven denitrification in a submerged sequencing batch biofilm reactor

Abstract

The ability of a biofilm to store influent organic carbon for subsequent use in the reduction of nitrate was investigated. A submerged biofilm, fed alternately with a high carbon feed and a high nitrate feed, was grown. Within four months of operation, stable and reproducible storage driven denitrification activity was observed. As much as 80% of influent acetate was usefully stored by the culture as poly-β-hydroxybutyrate (PHB) with limited oxygen entry (reactor $k_La < 2\ h^{-1}$). The stored electron donor could subsequently be used for effective denitrification. With this level of reducing power storage, this reactor could potentially remove all nitrogen from wastewaters with a feed C/N ratio of 3.6 kg COD/kg $N\text{-}NO_3^-$. Due to the lack of exclusion of oxygen during the denitrification phase, the carbon requirement for nitrogen removal observed was more typically 4.7 kg COD/kg $N\text{-}NO_3^-$. The reactor described offers an improvement over previous storage driven denitrification sequencing batch reactors (SBR) as it reduced the release of untreated ammonia retained in the settled sludge.

2.1 The need for improved carbon management for nitrogen removal

Complete biological nitrogen removal (BNR) from wastewater requires both nitrification and denitrification. Oxygen supply in the initial stage of wastewater treatment enables nitrification, but also oxidises available chemical oxygen demand (COD), limiting electron donor availability for denitrification. To allow sufficient

denitrification, additional carbon may need to be added, usually achieved either by addition of methanol, which increases cost of operation [54] or raw wastewater, which can cause ammonia release [37], compromising cost and efficiency.

An alternative approach to supply electron donor for denitrification may be the storage of influent COD within the cell as polymeric material, such as poly hydroxyalkanoates (PHA). PHA is produced by the uptake and storage of COD, such as organic acids, and can be used as the electron donor for denitrification [37, 51, 54]. Where acetate is the primary carbon source, the dominant PHA is poly-β-hydroxybutyrate (PHB) [28].

A large proportion of multistage wastewater treatments systems based on the physical separation of aerobic nitrification biomass from denitrification biomass developed to date use suspended culture sequencing batch reactor (SBR) technology for at least one of the nitrogen removal reactors [37, 40, 51, 54]. As SBRs retain a fraction of reactor liquor, the performance of multistage processes with denitrification in an SBR, will be limited by the proportion of retained nitrogen [40]. In the systems described, if the settled volume is 50%, then 50% of influent ammonia will be retained, limiting nitrogen removal to less than 50%.

In order to overcome this limitation, the storage denitrification reactor design should allow complete drainage such as in a biofilm. In addition, the use of a biofilm may also eliminate the need for settling and clarification of treated wastewater. The aim of this paper was establish and test a storage driven denitrification biofilm reactor with high storage and denitrification capacity for its capacity to overcome the drainage limited nitrogen removal previously reported.

2.2 Materials and Methods

2.2.1 Synthetic wastewaters used in this study

The synthetic wastewater solutions employed in this study were all based on the published laboratory work of Third *et. al.* [29] investigating SND in suspended growth SBRs. Modifications were made, as detailed below and in the relevant experimental sections, to alter the C/N ratio of the feed tested.

A primary treated wastewater from a local municipal wastewater treatment plant (Woodman Point Wastewater Treatment Plant) was substantially analysed to ensure the relevance of the chosen synthetic wastewater. Although the volume of influent wastewater varies with time, there was little variation in the average composition (Figure 2.1). The average measured composition of the wastewater compares well with published data [20] of the same plant (Table 2.1). Acidification of samples to suspend biological activity increased the amount of soluble COD (Figure 2.2) and as a result, samples were either filtered (0.45 μm syringe filter) or centrifuged (5 minutes at 1350 rpm) rather than acidified.

Acetate uptake phase feed composition

The composition of the synthetic wastewater used was based on established laboratory work investigating SND [29]. The carbon content was reduced to allow testing of the system with low C/N ratio wastewaters. The standard composition of the influent

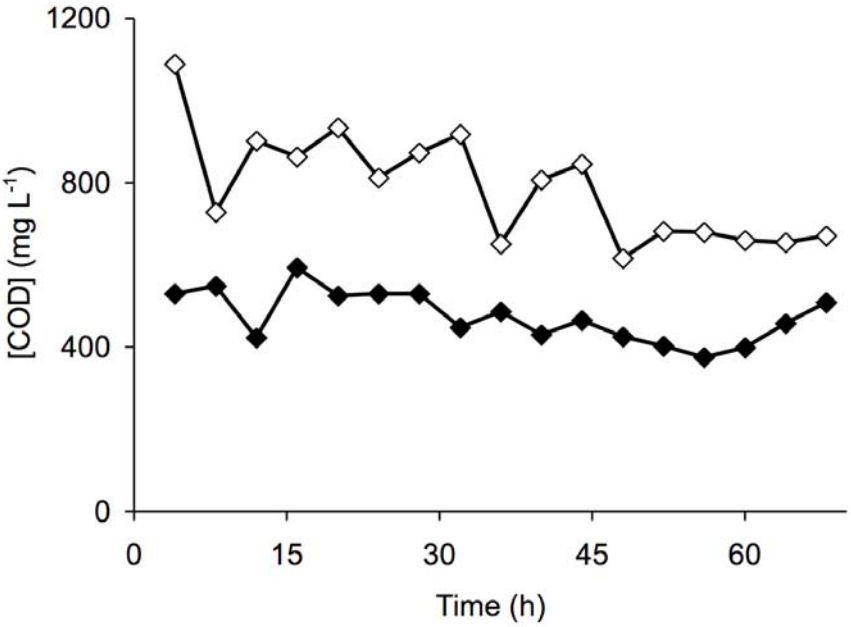

Figure 2.1 Variation of chemical oxygen demand concentration of wastewater entering the secondary treatment facility at the Woodman Point Wastewater Treatment Plant, Perth Western Australia. Filtered (◆) and unfiltered (◇) samples.

Table 2.1 Average composition of primary treated municipal wastewater at the Woodman Point Wastewater Treatment Plant, Perth Western Australia.

Chemical species	**Average amount in primary treated wastewater**	
	This study	**Bagg *et al* [20]**
Alkalinity	297 ± 6 mg $CaCO_3$ L^{-1}	282 mg $CaCO_3$ L^{-1}
COD – filtered	472 ± 62 mg COD L^{-1}	514 mg COD L^{-1}
COD – unfiltered	769 ± 111 mg COD L^{-1}	NA
Phosphate	0.50 ± 0.09 mM	0.42 mM
Ammonia	3.05 ± 0.30 mM	3.39 mM
Nitrate	Less than 1% total inorganic N	NA
Nitrite	Less than 0.1% total inorganic N	NA
Acetate	1.57 ± 0.24 mM	NA
Total VFA	1.66 ± 0.37 mM	NA
Average C/N ratio	11.1 ± 3.2 kg COD/kg N-NH_3	10.8 kg COD/kg N-NH_3

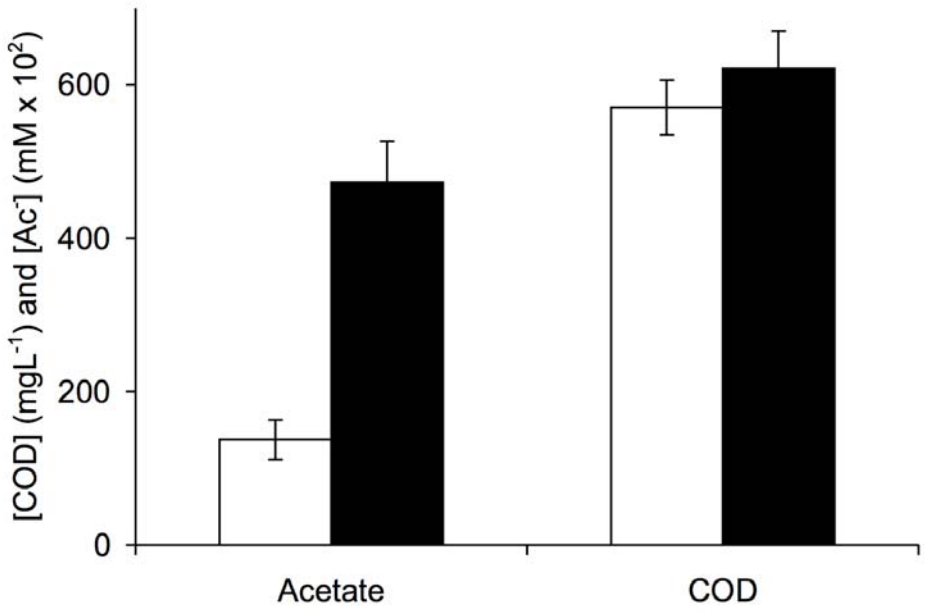

Figure 2.2 Effect of pre-treatment of wastewater samples to minimise microbial activity on the initial nutrient concentrations analysed. Samples were filtered (white bars) or acidified (10 mL samples acidified with 1 mL of 2.5 M H_2SO_4) (black bars).

synthetic wastewater was (mg L^{-1}) CH_3COONa 200, NH_4Cl 160, KH_2PO_4 44, $NaHCO_3$ 125, $MgSO_4.7H_2O$ 25, $CaCl_2.2H_2O$ 300, $FeSO_4.7H_2O$ 6.25, yeast extract 50 and 1.25 mL L^{-1} of trace element solution. Where different concentrations of nutrients were used, these are specified in the appropriate results section. The autoclaved trace element solution contained (g L^{-1}) EDTA 15, $ZnSO_4.7H_2O$ 0.43, $CoCl_2.6H_2O$ 0.24, $MnCl_2.4H_2O$ 0.99, $CuSO_4.5H_2O$ 0.25, $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.3, $NiCl_2.6H_2O$ 0.19, $NaSeO_4.10H_2O$ 0.21, H_3BO_3 0.014 and $NaWO_4.2H_2O$ 0.05 [29]. Although the concentration of COD is lower than in the real wastewater, this was acceptable as the purpose of this study was to develop a system with substantially better carbon conservation.

Denitrification phase feed composition

The standard composition of the denitrification feed was (mg L^{-1}) $NaNO_3$ 128, KH_2PO_4 44, $NaHCO_3$ 125, $MgSO_4.7H_2O$ 25, $CaCl_2.2H_2O$ 300, $FeSO_4.7H_2O$ 6.25, and 1.25 mL L^{-1} of trace element solution, as described above. There were variations in concentration of particular nutrients throughout the study and these are specified where relevant.

Biomass wash solution

A biomass wash solution was made that contained all of the same components at the same concentration as the synthetic wastewater except acetate, yeast extract, ammonia and phosphate.

2.2.2 Laboratory reactor design and operation

A storage driven denitrification submerged biofilm reactor (A) was set up with a recycle vessel (B) (Figure 2.3). The column reactor (A), with internal volume of 2L, was filled with a packing material (AMB^{TM} Biomedia Bioballs with total surface area of 850m^2/m^3 bioball volume and approximate active surface of 500m^2/m^3) for growth and support of the denitrifying organisms. The culture was seeded with return activated sludge from a local wastewater treatment plant and fed alternately a carbon feed (Section 2.2.1) and nitrate feed (Section 2.2.1) in order to encourage the growth of those organisms capable of both storage of carbon and denitrification using stored carbon. At the end of each phase, the effluent was pumped out as waste. The liquid volume of the

whole system could be adjusted by changing the volume of the recycle vessel and details are given where appropriate.

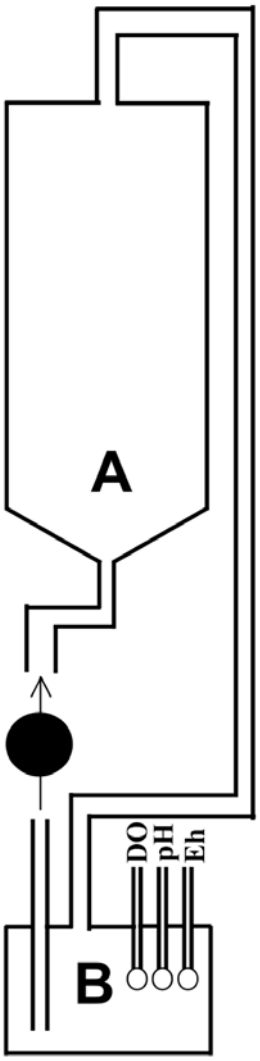

Figure 2.3 Storage driven denitrification reactor with a biofilm chamber (A) and liquid recycle vessel (B). The reactor was operated in two phases. The first was an acetate uptake phase fed a high acetate, high ammonia feed. At the end of the phase, the reactor was drained and then refilled with a nitrate rich feed. The recycle pump is indicated by a solid circle with arrow for direction of flow. Probes for pH, Eh and DO were located in the recycle vessel.

The recycle vessel, with between 0.350 L and 0.950 L capacity, was fitted with a dissolved oxygen probe, pH probe and an oxidation-reduction potential (ORP) probe, which were monitored continuously online with data logging (National Instruments, LABVIEW) into a spreadsheet.

The reactor was operated continuously with cycles of 360 minutes, with two distinct phases unless otherwise stated in the results. Phase one (130 minutes), in which acetate storage occurred, consisted of a rapid fill phase (2 – 5 minutes) with pumping into the column as well as the recycle vessel, carbon uptake phase and decant (4 – 8 minutes). The subsequent nitrate removal phase (230 minutes) consisted of a rapid fill phase (2 – 5 minutes), denitrification and decant (4 – 8 minutes) of the treated wastewater. The decant phases were assumed to achieve complete drainage of the biofilm.

2.2.3 Small scale shake flask tests

Biomass samples were taken from the storage driven denitrification biofilm at the end of the denitrification phase to ensure the lowest amount of stored PHB (Section 2.2.2). The biomass was removed from the bioballs by mixing in the wash solution (Section 2.2.1), pelleted by centrifugation (5 minutes at 1350 rpm) and resuspended in the wash solution. The subsequent biomass suspension was oxygenated for 30 minutes to ensure the removal of all residual bioavailable PHB and deoxygenated by purging with nitrogen.

All experiments were carried out in 50 mL anaerobic shake flasks containing 20 mL of prepared deoxygenated medium as specified below. At time 0, a 4 mL aliquot of prepared biomass suspension was added, mixed thoroughly and a 4 mL aliquot of mixed liquor taken for analysis. The biomass concentration after the time 0 sample was taken was 7.7 gL^{-1} in all tests. After an appropriate incubation length (at least 180 minutes) a

mixed liquor sample was taken for analysis. All tests were conducted in at least duplicate.

The medium used in the shake flasks differed with the purpose of each test. The initial tests were conducted with control flasks, with wash solution used as a medium and test flasks, with synthetic wastewater as medium (Section 2.2.1). Additional tests were conducted in which ammonia was not supplied in the medium.

A final set of experiments was performed in which the suspension of biomass was given a different pre-treatment prior to incubation. Pre-treatments were:

- Pre-treatment 1: deoxygenated without prior oxygenation. Deoxygenation achieved by 30 minutes of nitrogen bubbling. Samples were then incubated under anaerobic conditions.
- Pre-treatment 2: oxygenated without a subsequent deoxygenation. Oxygenation achieved by 30 minutes of air bubbling. Samples were then incubated under anaerobic conditions.
- Pre-treatment 3: neither oxygenated nor deoxygenated. In pre-treatment 3, the biomass was introduced into a shake flask with deoxygenated medium at time zero at which point the flask was left open. After mixing for 10 minutes, the headspace was degassed with nitrogen and the tube sealed. Pre-treatment 3 was designed to mimic more closely the conditions in the laboratory scale reactor.

2.2.4 Chemical analysis

Nitrogen compounds

Most nitrogen compounds were monitored spectrophotometrically. The nitrate analysis method was adapted from Third *et al* [29]. Samples of 100 μL, containing 0.1 to 3 mM nitrate, were treated with 25 μL saturated ammonium amidosulphonate in deionised water to remove nitrite and then allowed to react for 10 minutes with 300 μL 5% (w/v) sodium salicylate in 98% sulfuric acid. After addition of 3 mL ice cold 4M sodium hydroxide, mixing and cooling, the samples were measured at 420 nm. It was essential that the sodium hydroxide was cold to minimise heat evolved from the exothermic reaction.

Nitrite was analysed by the formation of an azo dye. For analysis, 1 mL samples, containing 0.002 to 0.05 mM nitrite, were allowed to react for 30 minutes with 1 mL 1% (w/v) sulphanilic acid in 1 M hydrochloric acid and 1 mL 0.1% (w/v) N-1-naphthylethyldiamine dichloride in water and measured at 540 nm [55].

For ammonium analysis, 2 mL samples, containing 0.01 to 0.2 mM ammonium, were pretreated with 25 μL each of mineral stabiliser and polyvinyl alcohol dispersing agent (supplied by Hach™) to inhibit precipitation of calcium, magnesium, iron and sulphide [37]. The samples were then analysed using the Nesslerisation method [56] by addition of 100 μL of Nessler's reagent, allowed to react for exactly 1 minute and measured at 425 nm.

Dissolved nitrous oxide was analysed by gas chromatography [57]. In order to inhibit biological activity, 2 mL liquid samples were treated with 100 μL of 1 M sulphuric acid, sealed, equilibrated and analysed by gas chromatography (Table 2.2).

Table 2.2 Gas chromatograph parameters used for nitrous oxide analysis.

Parameter	Description
Instrument	Hewlitt Packard 5890 Series II Gas Chromatograph
Column	2.0 m stainless steel, 2.0 mm internal diameter, Porapak QS packed column
Injection port temperature	250°C
Oven temperature program	2 min at 40°C
	Temperature ramp at 50°C/min to 120°C
	4 min at 120°C
	Cooled to 40°C
Detector	Electron capture detector
Detector temperature	300°C

Phosphate determination

Soluble phosphate was analysed using the ascorbic acid method [56]. A 2 mL sample, containing between 0.01 and 0.04 mM orthophosphate, was treated with 320 μL of ascorbic acid reagent, allowed to react for at least 10 and not more than 30 minutes and measured at 880 nm. The ascorbic acid reagent consisted of (for 10 mL) 5 mL of 2.5 M sulphuric acid, 0.5 mL 8.21 mM potassium antimonyl tartrate, 1.50 mL 32.37 mM ammonium molybdate and 3 mL 99.91 mM ascorbic acid. Each solution was added in the order listed, at room temperature with mixing between and left to briefly equilibrate prior to use. Once mixed, the reagent was stable for 4 hours.

Carbon determination

Both volatile fatty acid (mainly acetate) concentration in the recycle liquid and PHB content of the lyophilised biofilm was measured by GC using a Varian Star 3400 Gas Chromatograph with flame ionisation detector (Table 2.3). Acetate samples were acidified to ensure the molecular form was dominant for passage through the GC column. 900 μL samples were treated with 100 μL 10% (v/v) formic acid in deionised water.

Table 2.3 Gas chromatograph parameters used for acetate and PHB analysis.

Parameter	Description	
	VFA (acetate) analysis	PHB analysis
Instrument	Varian Star 3400 Gas Chromatograph	
Column	15 m Alltech EC-1000 capillary column with internal diameter 0.53 mm and film thickness 1.2 μm	
Injection port temperature	200°C	
Oven temperature program	Initial temperature 80°C	Initial temperature 80°C
	40°C/min to 140°C	35°C/min to 152°C
	1 min at 140°C	4°C/min to 165°C
	50°C/min to 230°C	15°C/min to 220°C
	2 min at 230°C	13 min at 220°C
	Cooled to 80°C	Cooled to 80°C
Detector	Flame ionisation detector	
Detector temperature	250 °C	

PHB and glycogen analysis was performed with single, large (~1 g) samples of biomass from the reactor from randomised bioballs (~10). The large samples were freeze dried and then ground in a mortar. Triplicate samples were then taken for PHB and glycogen

analysis as required (see details below). The triplicate samples were treated as required and then measured in triplicate, that is, for example, three injections were made of each sample to the GC for PHB analysis.

PHB content of lyophilised biomass was extracted, hydrolysed and esterified in a one step closed reflux [58]. Up to 20 mg of biomass was placed in a sample tube to which is added 1.45 mL of a 1:4 mixture of concentrated hydrochloric acid and 1-propanol. 50 μL of 20 mg mL^{-1} benzoic acid was used as an internal standard. An additional 1.5 mL of dichloroethane was added before sealing and refluxing at 150°C for 2 hours with regular shaking. The organic phase was extracted with deionised water to remove free acids, dried over sodium sulphate and analysed by GC (Table 2.3) [37]. Hydroxybutyric acid standards, containing 1 to 5 mM hydroxybutyrate, were prepared from the sodium salt of L-(+)-β-hydroxybutyric acid and treated as above. PHB concentration in the reactor was expressed as percentage of volatile solids (VS). The presence of PHB was confirmed qualitatively using Sudan Black staining [59] (Section 2.2.5).

Glycogen was extracted and digested in a one step reflux of up to 100 mg biomass samples in 5 mL of 0.6 M hydrochloric acid at 100°C for 5 hours with regular shaking [60]. The supernatant was collected and measured by glucose analysis using a YSI 2700 Select Biochemistry Analyser fitted with a dextrose membrane for D-glucose analysis and/or by a specific enzymatic assay using glucose oxidase, peroxidase and ABTS [61]. Larger biomass sample sizes caused underestimation of glycogen content in replicates of identical biomass samples.

As a defined carbon source was generally used (Section 2.2.1), specific analysis techniques were used to determine these where appropriate. Where an undefined carbon

source was provided, the more general measure of COD was used. A closed reflux, colorimetric method was chosen to determine COD, as described in Greenberg [56]. Two reagent solutions were required for this analysis. The digestion solution contained 10.216 g of dried $K_2Cr_2O_7$, 167 mL of concentrated sulfuric acid and 33.3 g of $HgSO_4$ per litre of reagent. The sulfuric acid reagent contained 5.5 g of $AgSO_4$ per kg of concentrated sulfuric acid. The silver was provided to minimise interference from ammonia oxidation. To 1.5 mL of sample in a sealable glass tube, 0.9 mL of the digestion solution and 2.1 mL of the sulfuric acid reagent were added in sequence very carefully to minimise mixing. Once all the reagents were added, the tube was sealed and mixed. It was then allowed to reflux, with regular mixing, at 150°C for 2 hours before cooling to room temperature and reading the absorbance at 600 nm. A solution prepared from dried potassium hydrogen phthalate was used to generate a standard curve in the region of 0 to 950 mg COD/L. As very low levels of nitrite (< 0.15 mM NO_2^-) were found in the samples where COD analysis was employed, the addition of sulfamic acid as described in Greenberg [56], to eliminate interference from oxidation of this anion was not considered necessary. COD analysis of the Woodman Point Wastewater Treatment Plant samples above revealed that treatment with acid to cease biological activity in the samples increased the amount of soluble COD (Section 2.2.1). All samples were subsequently centrifuged (5 minutes at 1350 rpm) and/or filtered (0.45 μm syringe filter) to remove biological material.

Inorganic carbonate content and more generally, total alkalinity or buffer capacity, was measured as described in Greenberg [56] with titration of samples to pH 4.5 with a standardised hydrochloric acid solution. The endpoint was determined either visually with bromocresol green indicator or by potentiometric measurement. The choice of

endpoint reflected the mixed contribution to alkalinity in the samples rather than solely carbon dioxide.

Biomass determination

Prior to each experiment, the bioballs in the column reactor were randomised. The growth of the biofilm was uneven across individual bioballs and as a result, all biomass samples taken were large (10 bioballs each with ~0.1 g for a total sample of ~1 g) to approach some level of representativeness. However, it was possible that at different locations along the column different environmental conditions were experienced and therefore the biomass samples were taken as semi quantitative support for evidence accumulated elsewhere. Total solids (TS) and volatile solids (VS) were measured for each biomass sample taken by drying at 60°C for 24 h and ignition at 500°C to constant weight respectively [56].

2.2.5 Microscopic examination of biomass

Samples of biomass from each reactor were routinely examined microscopically. A number of stains were employed to elicit more information regarding metabolism and characterisation of the mixed cultures [25]. All samples for staining were air dried thoroughly before the stain was applied. Direct observation was also made of fresh wet mounted biomass samples.

Poly hydroxyalkanoate stain

The presence of poly hydroxyalkanoate energy storage granules within cells was observed with Sudan Black staining. Solution 1 was prepared from 0.3% (w/v) Sudan Black in 60% ethanol. Solution 2 was prepared from 0.5% (w/v) Safranin O in distilled water. Air dried slides were stained for 10 minutes with solution 1, maintaining dampness with further addition of solution 1 if required, and rinsed with water for 1 second. Slides were then stained for 10 seconds with solution 2, rinsed with well water and blotted dry. Slides were examined under oil immersion at 1000X with direct illumination. Intracellular granules of PHA appeared blue-black and cell cytoplasm was clear or pale pink.

2.2.6 Calculations

Determination of oxygen transfer coefficient and oxygen uptake rate

The only locations through which oxygen could be transferred to the system were at the liquid air interface in the recycle vessel and through the tubing connecting the recycle vessel to the column reactor. The volumetric mass transfer coefficient (k_La) of oxygen into the vessel was determined experimentally by the operation of the reactor with inert packing material to simulate the liquid load capacity routinely in operation [62]. The reactor was filled with deionised deoxygenated water and the increase in dissolved oxygen monitored. The k_La of the system was found to be 1.06 ± 0.05 h^{-1}, considerably lower than systems with formal oxygen delivery. k_La was determined mathematically as

the negative slope of the curve resulting from plotting the oxygen transfer rate against dissolved oxygen concentration [62].

The oxygen uptake rate (OUR) of the culture was then calculated dynamically assuming steady state conditions using the concentration of oxygen in the liquid phase (c_L) and the saturating concentration of oxygen in the gas phase (c_S) as follows:

$$OUR = k_L a.(c_S - c_L) \qquad \text{Equation 2.1}$$

The total amount of oxygen that is consumed in each cycle is then calculated by the integral of the OUR with respect to time.

C/N ratio of removal achieved

The observed carbon requirement for nitrogen removal (C/N ratio of removal) observed in each cycle was calculated based on the amount of nitrogen (in this case expressed as kg N-NO_3^-) that was reduced during the denitrification phase relative to the amount of carbon (acetate, expressed as kg COD; Appendix A) that was removed in the acetate uptake phase as shown below:

$$C/N = \frac{acetate_{removed}(kgCOD)}{nitrogen_{removed}(kgN)} \qquad \text{Equation 2.2}$$

2.3 Results and Discussion

2.3.1 Can a Storage Driven Denitrification culture be successfully cultivated?

In order to conserve as much COD as possible for use as electron donor in denitrification, intracellular storage of influent acetate, possibly as PHB, needed to be achieved. Since a source of electron acceptor (nitrate) and donor (acetate) was not available to the culture simultaneously, the potential for establishing a bacterial population capable of storage driven denitrification using sequestered COD was investigated.

A sample of mixed liquor return activated sludge from a local wastewater treatment plant was obtained and inoculated into the column reactor (Section 2.2.2). The biomass was fed alternately with a COD rich feed and a nitrate rich feed (Section 2.2.1) to encourage those microorganisms capable of storage driven denitrification. The activity of the culture was tested after two days of operation, 4 weeks of operation and 4 months of operation.

After two days, the anaerobic storage capacity of the culture in response to the acetate feed was low (Table 2.4; 0.68 mM h^{-1}). Since the reactor k_La was also low (1.1 h^{-1}; Section 2.2.6) no more than 0.04 mM of influent acetate could have been oxidised (based on maximum amount of oxygen transfer at DO = 0 mg L^{-1}; similar calculation shown in Appendix B). The removal of acetate from solution during the acetate uptake phase was not related to subsequent denitrification activity as nitrate was not removed (Table 2.4).

Table 2.4 Change in nutrient removal rates in the storage driven denitrification reactor after inoculation.

Time after inoculation	Rate of acetate uptake	Loss of ammonia during acetate uptake phase	Rate of nitrate uptake
2 days	0.68 mM h^{-1}	0%	No detectable activity
4 weeks	2.3 mM h^{-1}	7%	1.1 mM h^{-1}
16 weeks	4.6 mM h^{-1}	35%	3.0 mM h^{-1}

After four weeks of operation, a substantial improvement in the rate of acetate uptake as well as detectable denitrification activity had occurred (Table 2.4). As no soluble source of COD was available during the denitrification phase, a COD storing denitrifying population was enriched. A further improvement and stabilisation was observed after four months of operation (Table 2.4). A reproducible response of acetate and nitrate removal in each phase was established (Figures 2.4 and 2.5).

After four weeks of operation, 7% loss of ammonia was observed during the acetate uptake phase while the concentration of ammonia increased during the denitrification phase. The loss of ammonia during the acetate uptake phase increased with the duration of operation (Table 2.4). The amount of ammonia removed during acetate uptake stabilised to an average of 22 ± 13%. Less ammonia seemed to be removed after removing excess biomass from the reactor. The nitrogen removal limitation due to retention of ammonia was improved over published suspended culture systems [40, 51]. Adsorption of ammonia to a biofilm has been observed in previous biofilm reactors [53].

Fate of nitrate during the denitrification phase

A satisfactory level of nitrate removal (> 90%) was achieved and stabilised with time as described above. However, it was not clear if reduction of nitrate to nitrogen gas was achieved. The anthropogenic contribution to environmental release of the greenhouse gases nitric and nitrous oxides is increasing [63]. If a wastewater treatment method contributes to the production and release of these gases on greenhouse gas accumulation and associated global warming, then it needs to be identified and minimised. It was necessary to characterise whether the nitrate removal occurring in the storage driven denitrification reactor was *via* reduction of nitrate to nitrogen gas or possibly nitrous oxide.

Transient concentrations of nitrite and nitrous oxide were detected during the denitrification phase (Figure 2.6). At the end of the denitrification phase, both were negligible (nitrite 4.8% and nitrous oxide 0.15% of remaining nitrogen). Both intermediates were observed to reach a peak concentration over time and the nitrite peak occurred (60 minutes into the denitrification phase) prior to the nitrous oxide peak (90 minutes into denitrification phase). The observation of both intermediates with sequential peak concentrations suggested that the complete reduction pathway of nitrate to nitrogen gas had been achieved. Each reduction reaction occurs with a lag in activation of the relevant organisms as has been observed previously [64, 65].

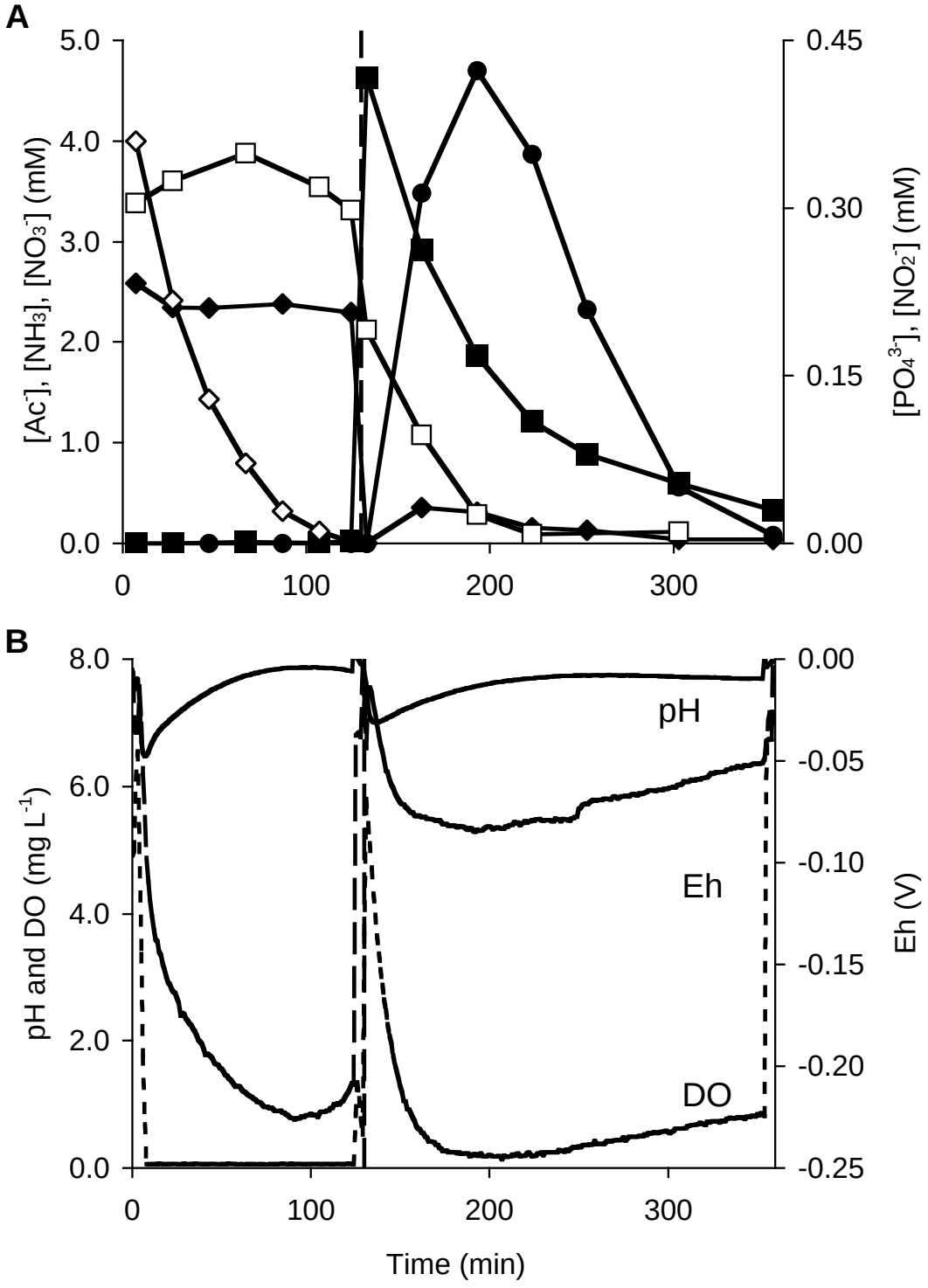

Figure 2.4 Typically observed metabolite behaviour during the storage driven denitrification operation in two phases, fed alternatively with batches of acetate and ammonia feed and then nitrate feed (Section 2.2.1). (Acetate uptake phase feed: Ammonia 2.9 mM and Acetate 4.0 mM; 6.4 kg COD/kg N-NH_3; Denitrification phase feed: Nitrate 4.6 mM): Acetate (◇), phosphate (□), ammonia (◆), nitrate (■) and nitrite (●). Vertical line indicates phase change from acetate uptake phase (130 minutes) to denitrification phase (230 minutes).

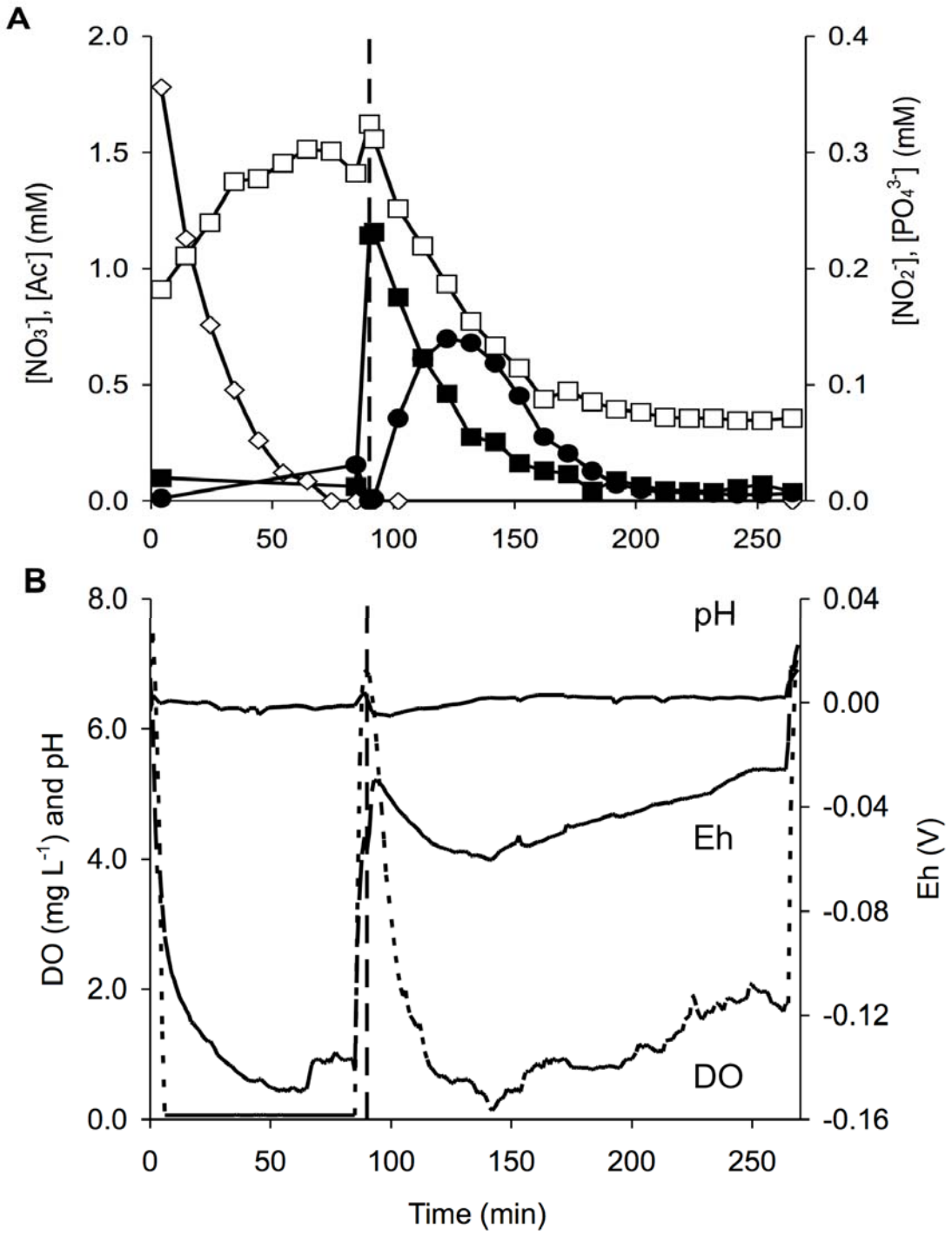

Figure 2.5 Reproducible typically observed metabolite behaviour during the storage driven denitrification operation in two phases, fed alternatively with batches of acetate and ammonia feed and then nitrate feed (Section 2.2.1). (Acetate uptake phase feed: Ammonia 2.0 mM and Acetate 1.8 mM; 4.1 kg COD/kg N-NH_3; Denitrification phase feed: Nitrate 1.2 mM): Acetate (◇), phosphate (□), ammonia (◆), nitrate (■) and nitrite (●). Vertical line indicates phase change from acetate uptake phase (130 minutes) to denitrification phase (230 minutes).

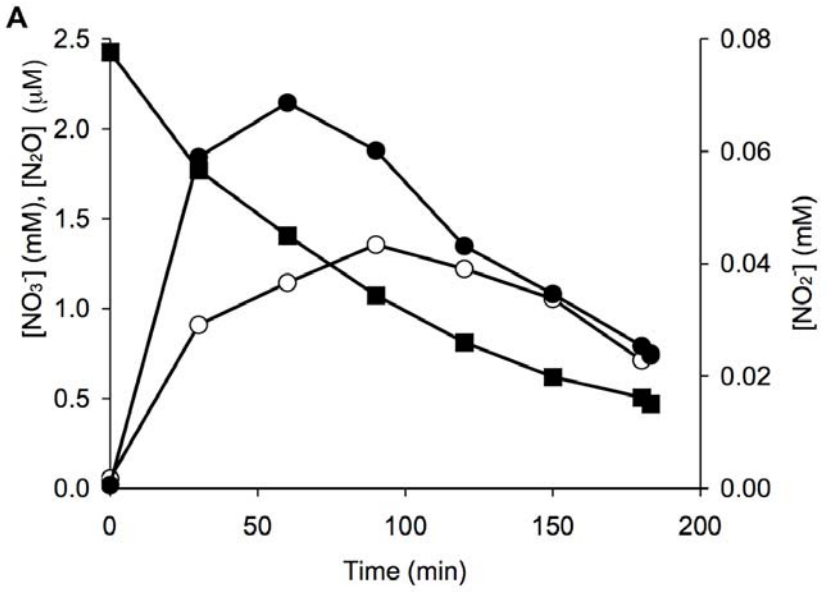

Figure 2.6 Time course during the denitrification phase of the storage driven denitrification biofilm reactor (Feed Nitrate 2.4 mM): Nitrate (■), nitrite (●) and nitrous oxide (○).

The supply of a nitrite rather than nitrate feed resulted in the reduction of nitrite using sequestered COD (Figure 2.7). Nitrite reduction was about five times faster than the reduction of nitrate as expected from literature (Table 2.5). According to this text, even at low concentrations, the nitrite reduction rate should be higher than that of nitrate reduction. While the data fit well with expected Michaelis-Menten kinetics, the high K_m values indicate that diffusion of substrate through the biofilm may have affected the rates observed. Under diffusion limitation, the observed kinetics of biological processes become a product of diffusion kinetics and microbial kinetics. Depending on the mixing of the system, the thickness of the biofilm and the concentration range of the substrate one of the two kinetic systems may predominate [19, 66]. For very low concentrations both systems may be similar (approaching first order kinetics). For rates at low concentrations and floc biofilms (e.g. activated sludge) the term apparent K_m has been used as being distinctly higher than the actual K_m of non attached single biomass cells. Analysis of the data in Table 2.5 indicates that apparent first order kinetics due to

diffusion were important to the performance of the biofilm (Table 2.6) although further investigation would be required to confirm the result. In addition to the more complex kinetics defined by diffusion (first order) and enzyme (Michaelis-Menten) processes, it can be useful to examine zero order kinetics [67] (known as half order kinetics) in biofilm processes. Large deviations between modelled data and real data tend to occur in systems modelled with the simplified zero order kinetic approach. As the first order or Michaelis-Menten modeled data provided apparent rate constant values with reasonably high correlation, the zero order kinetics was not explored in this project.

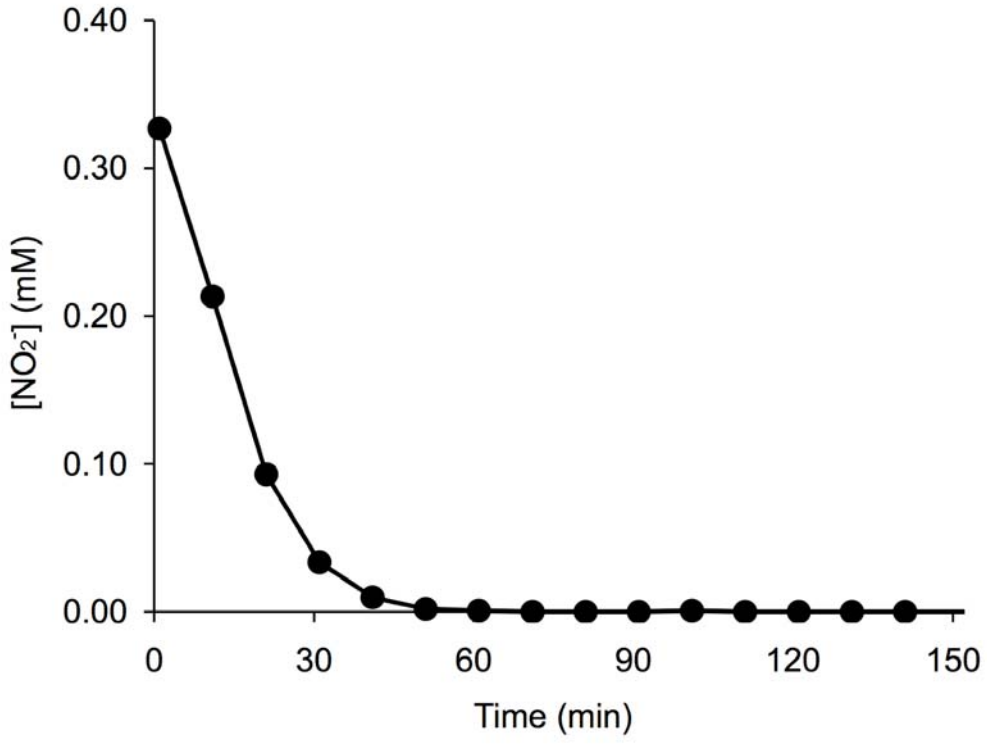

Figure 2.7 Removal of nitrite during the denitrification phase of the storage driven denitrification reactor fed a nitrite only feed (Nitrite 0.33 mM).

Table 2.5 Observed rate constants of nitrogen oxide reduction by the storage driven denitrification reactor.

Nitrogen substrate	Michaelis-Menten constant, K_m (R^2)[1]	Michaelis-Menten constant, K_m from literature[2]
Nitrate	4.5 mM (0.99)	0.3 – 3.8 mM
Nitrite	0.046 mM (0.98)	0.03 – 0.74 mM

Table 2.6 Observed rate constants of nitrogen oxide reduction by the storage driven denitrification reactor.

Nitrogen substrate	First order rate constant, k (R^2)[3]
Nitrate	0.38 h^{-1} (0.98)
Nitrite	5.3 h^{-1} (0.98)

These results indicate that a population of bacteria could be enriched which exhibited COD storage and the subsequent use of stored COD as electron donor for denitrification. The identity of the storage compound used for electron donor in denitrification as well as the possible yield from influent COD need to be further investigated.

[1] K_m value derivation (with data) shown in Appendix C.
[2] Literature data from Zumft [14]; a literature review of denitrification processes and enzymes.
[3] First order rate constant derivation (with data) shown in Appendix C.

2.3.2 What is the identity of the principle storage compound?

The build up of a storage driven denitrification process has been established (Section 2.3.1). While it is hypothesised that the principle storage compound for sequestration of acetate intracellularly was PHB due to the restriction of influent organic carbon to acetate, the above results do not necessarily prove this. It was necessary to establish if PHB was the main carbon storage compound in order to achieve a meaningful mass balance for the storage driven denitrification process.

Samples of biomass were taken from the reactor and treated with an excess of acetate (100 mM) to encourage accumulation of PHB. Samples were then microscopically examined for PHA content (Figure 2.8). The biofilm appeared to be dominant in a single morphology cell type although filamentous material, rich in PHA, was also detected.

Staining with Sudan Black indicated PHA material, which suggested that PHB was produced from acetate. Large biomass samples (~1 g) were taken for chemical analysis PHB (Section 2.2.4). During the acetate uptake phase, PHB increased from 0.20% to 0.27% and subsequently back to 0.19% during denitrification (Figure 2.9).

While the small change in intracellular PHB may seem insignificant, it must be remembered that the amount of biomass in the biofilm was very large (~ 50 g) relative to the amount of acetate fed (2.6 mM; 0.2 g) and so it was likely that the change detected would be small. The pattern of change of PHB (increase during acetate uptake and decrease during denitrification) was reproducibly detected in the storage driven denitrification biofilm (Figures 2.9 and 2.10). In experiments with an excess supply of

the electron acceptor nitrate (Figure 2.10), a small amount of PHB was not removed which suggests that at least some PHB was integral to cell structure as has been observed previously [29, 37].

The possibility of glycogen accumulation, especially given the near anaerobic nature of operation of the storage driven denitrification biofilm, needed to be explored. Glycogen measurements of the same experiment above revealed a stable glycogen level in the biofilm (Figures 2.9 and 2.10) This lack of glycogen involvement as a storage polymer was detected reproducibly (Section 5.3.5). It was hypothesised that glycogen content was principally part of the cellular and biofilm structure and not bioavailable for denitrification. PHB was concluded to be the major carbon storage polymer.

2.3.3 Carbon balance in the storage driven denitrification biofilm

While there was a clear relationship between removal of acetate from the bulk reactor liquor, production of PHB in the biomass during the acetate uptake phase and subsequent reduction in PHB content during denitrification, a mass and electron balance was not available. An accurate mass balance attempt was limited by the ability to take a representative sample of biomass.

In order to stay independent of biomass sampling, a surrogate electron balance was attempted by supplying a substantial excess of nitrate to the reactor in the denitrification phase in order to assess the total amount of effective reducing power stored during the acetate uptake phase. The amount of nitrate reduced in this experiment should be linked directly to the usage of carbon sequestered from the preceding acetate uptake phase.

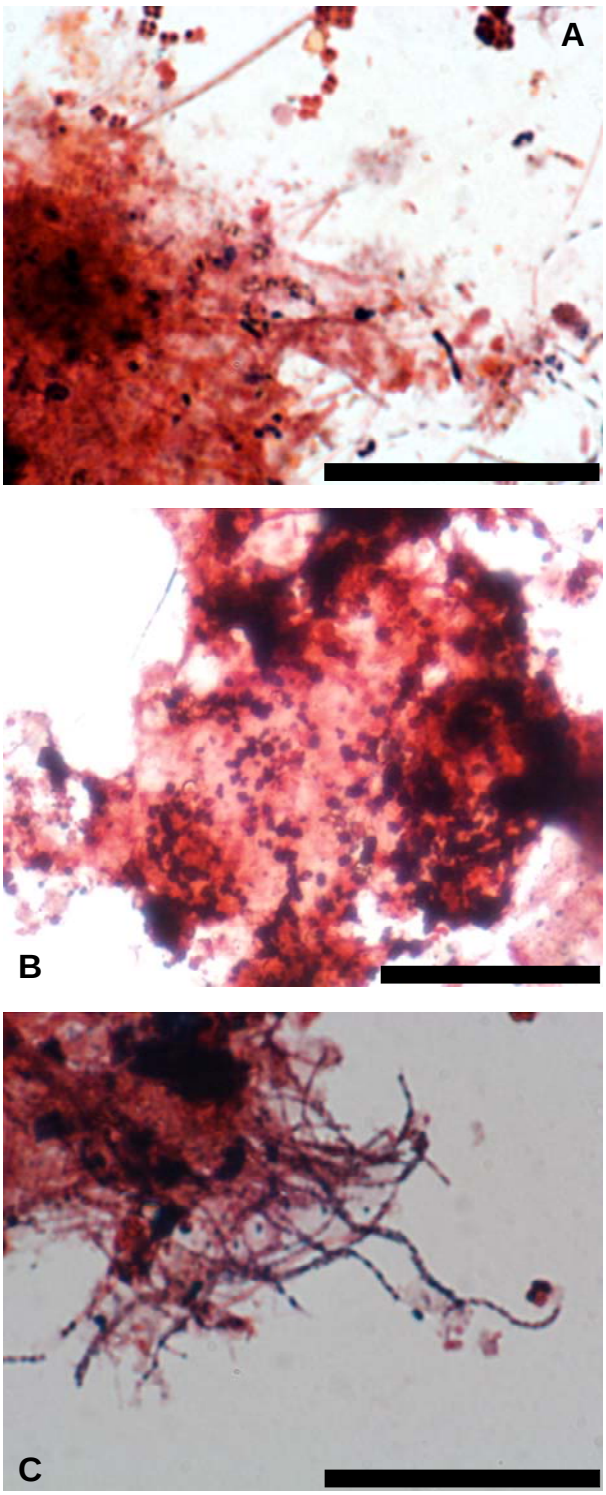

Figure 2.8 Sudan Black stained (Section 2.2.5) samples of biofilm material initially (A: 100 × 1.6 magnification, size bar 25 μm), after 3 hours (B: 40 × 1.6 magnification, size bar 50 μm) and 4 hours (C: 100 × 1.6 magnification, size bar 25 μm) incubation with 100 mM acetate. Cell cytoplasm revealed as red/pink material and PHA inclusions appear as blue/black granules. Image C shows filamentous material in the biofilm.

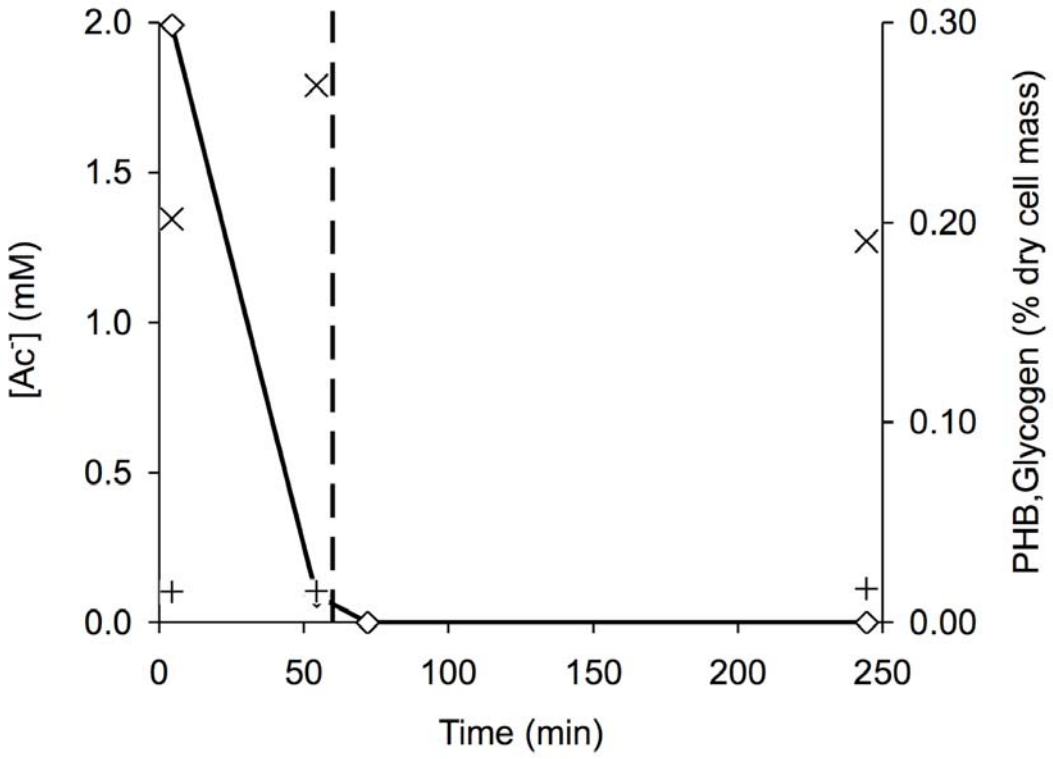

Figure 2.9 Observed change in carbon compounds during the storage driven denitrification process. Feed during acetate uptake phase: 2.0 mM acetate and 1.8 mM ammonia, C/N ratio 5.0 kg COD/kg N; Feed during denitrification phase: 1.0 mM nitrate. Acetate (◇), glycogen (+) and PHB (×). Vertical dashed line indicates change of phase from acetate uptake phase (60 minutes) to denitrification phase (190 minutes).

After a standard acetate uptake phase with low oxygen transfer (k_La 1.1 h^{-1}) where 2.0 mM of acetate was supplied and completely removed (Figure 2.10), an excess of nitrate (4.2 mM) was supplied of which 2.0 mM nitrogen was reduced. An extended phase length (350 minutes) was used to allow consumption of all available PHB.

From the assumption that 1.6 moles of nitrate can be reduced per mole of acetate (Equation 1.8), it could be calculated that 61% of acetate supplied was stored as PHB and subsequently used for denitrification (Appendix B). This was comparable to that

observed under limited oxygen supply conditions for SND cultures [37]. The C/N ratio of removal for this experiment was 4.7 kg COD/kg N, which compares well with literature estimates of practicably possible nutrient removal achievement [19, 37] where up to 7 kg COD/kg N is required for nitrogen removal. Due to the low, but measurable levels of oxygen entering the system during the acetate uptake phase, it was also possible to estimate the amount of acetate that was oxidised by oxygen during that phase. This was found to be 11% of the influent acetate (Appendix B).

The consequence of the low oxygen entry characteristics of the reactor system was that a small amount of oxygen was also available during the denitrification phase and may have contributed to the oxidation of intracellular PHB. Due to a gradient of nutrients along the axis of liquid flow [68], those microbes in the bottom of the reactor were expected to have the highest exposure to oxygen and therefore wasted the most COD. This additional supply of oxygen during denitrification accounted for an additional 19% (Appendix B) of stored substrate consumed. Considering the above electron donor loss to oxygen, the actual storage amount observed was 80% of the influent electron donor supply, a very competitive conservation of native COD. The remainder of electron donor content, 9%, was likely to be used for assimilation. If oxygen delivery is prevented during denitrification, this is likely to remove all nitrogen in a wastewater with a C/N ratio of 3.6 kg COD/kg N, approaching the stoichiometric minimum (2.9 kg COD/kg N; Appendix A) and superior to many literature sources (Section 2.3.4).

The very low amount of assimilation estimated in the SDDN reactor is consistent with other studies where storage of influent COD was maximum [37, 50]. Additional COD for assimilation could have been supplied through the degradation of dead cells in the biofilm, which would also contribute towards a steady state biofilm.

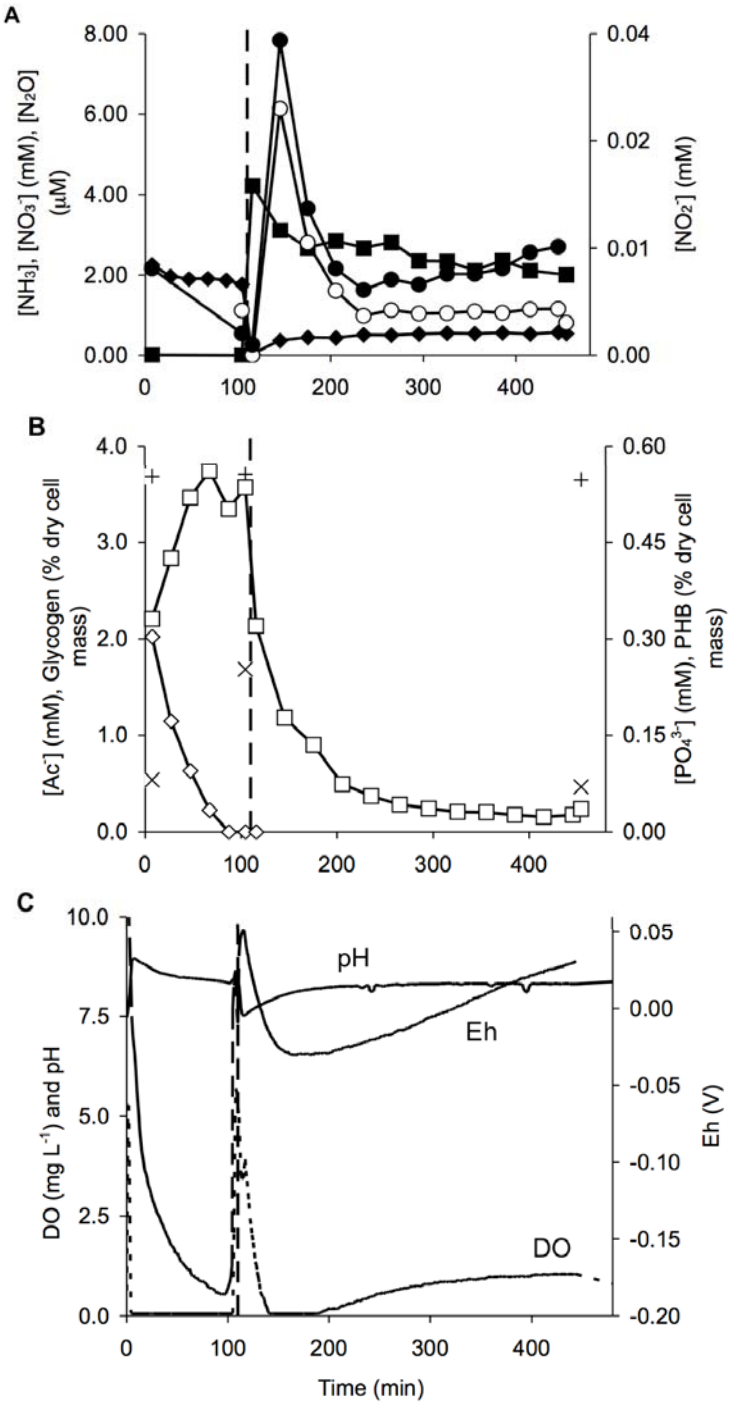

Figure 2.10 Effect of an excess nitrate feed during the denitrification phase after a standard acetate uptake phase. Feed during acetate uptake phase: 2.0 mM acetate and 2.3 mM ammonia, C/N ratio 4.1 kg COD/kg N; Feed during denitrification phase: 4.2 mM nitrate. Ammonia (◆), nitrate (■), nitrite (●), nitrous oxide (O), acetate (◇), phosphate (), glycogen (+) and PHB (×). Vertical dashed line indicates change of phase from acetate uptake phase (110 minutes) to denitrification phase (350 minutes).

The amount of storage observed in the excess nitrate test indicates a carbon balance of 0.71 Cmoles of PHB produced per Cmole of acetate consumed (Figure 2.11 and Table 2.7). Under oxygen limiting conditions (DO 0 mgL^{-1}; k_La 6 h^{-1}), Third *et al* [29] observed 0.63 Cmoles of PHB produced per Cmole of acetate. The higher yield of PHB in the storage driven denitrification reactor under lower oxygen supply conditions than in Third *et al* [29], was expected as it had been previously observed that decreased oxygen supply increased the yield of PHB [29].

Confirmation of electron balance in small scale tests

Small scale suspended biomass shake flask tests (Section 2.2.3) were conducted to find a carbon mass balance using an alternative approach based on accumulation of PHB from consumed acetate. Similar trends in all the small scale experiments conducted were observed with respect to acetate, PHB and glycogen (Figure 2.12) confirming the carbon balance observed in the laboratory scale reactor in the excess nitrate experiment above, that is 0.65 Cmoles of PHB produced per Cmole of acetate (Figure 2.12; Table 2.7).

Interestingly, the removal of acetate in the bulk liquor, not only related to an increase in PHB but also a reduction of glycogen. This indicates that glycogen may be a source of energy for the uptake and conversion of acetate into PHB under anaerobic conditions. This trend was not detected in the nitrate excess experiment where glycogen was clearly not involved in metabolism (Figure 2.10 and Table 2.7).

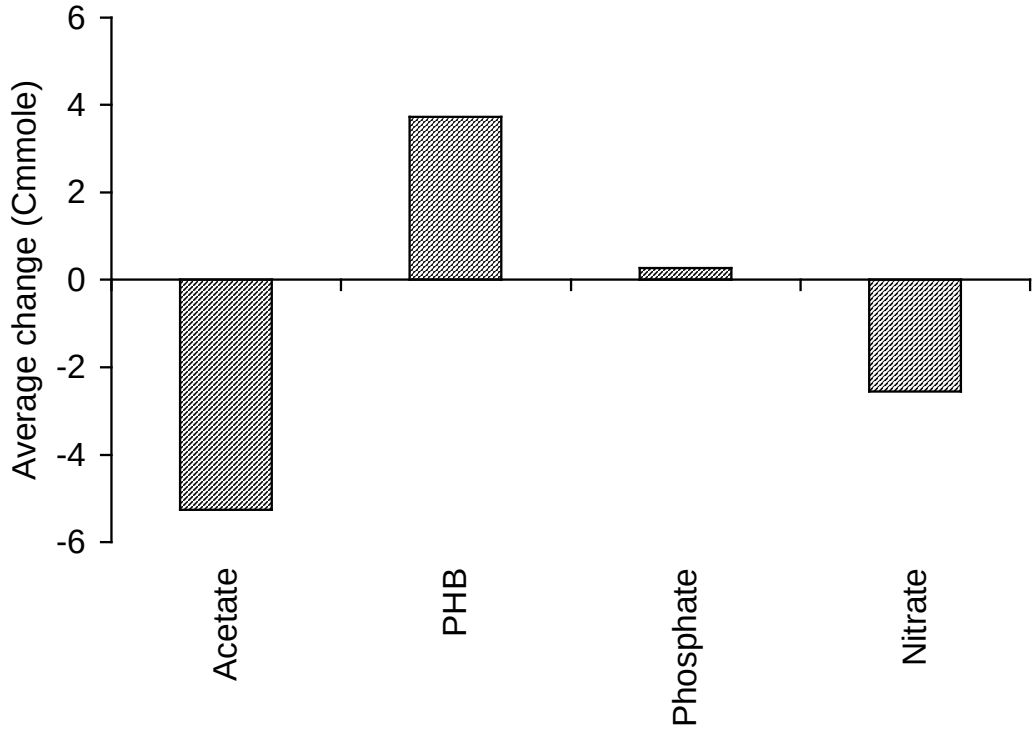

Figure 2.11 Change in metabolite (Cmmole) during the acetate uptake phase (acetate, PHB and phosphate) and denitrification (nitrate) in the excess nitrate fed experiment. Feed during acetate uptake phase (110 minutes): 2.0 mM acetate and 2.3 mM ammonia, C/N ratio 4.1 kg COD/kg N; Feed during denitrification phase (350 minutes): 4.2 mM nitrate. PHB is calculated based on nitrate reduced[4].

Table 2.7 Electron balance for production of PHB from acetate and consumption of glycogen in the excess nitrate fed laboratory scale experiment and small scale shake flask[5] experiments.

Experiment	PHB produced from consumed acetate	Glycogen consumed during acetate uptake
	(Cmmole PHB/Cmmole Ac)	**(Cmmole Glycogen /Cmmole Ac)**
Excess nitrate	**0.71**	**0**
Shake flask	**0.65**	**1.22**

[4] See Appendix B for calculation.

[5] The small scale shake flask experiments are discussed below.

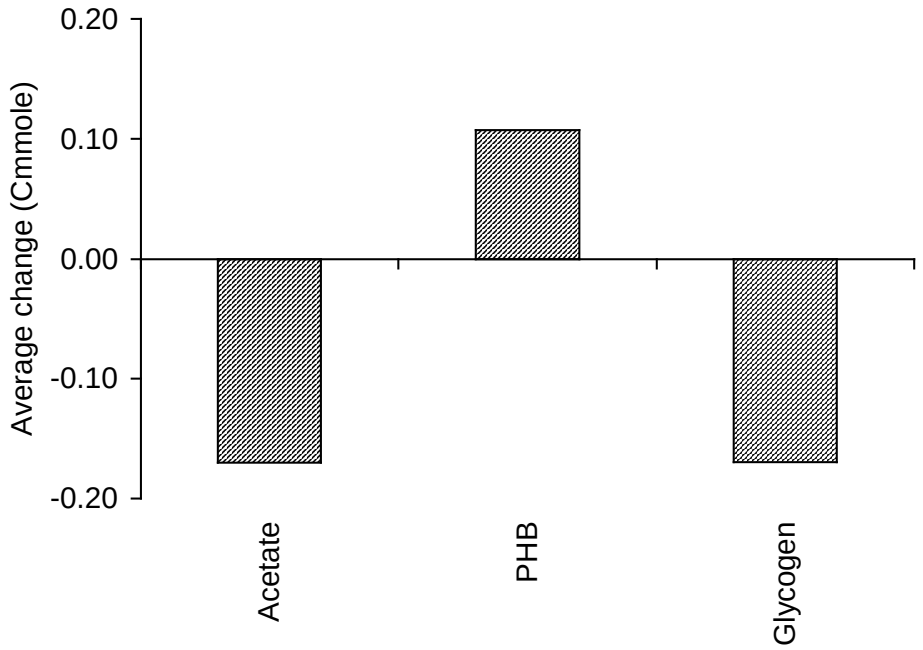

Figure 2.12 Change in metabolite (Cmmole) in storage driven denitrification biomass removed from bioballs by washing and incubation (180 minutes) in 20 mL medium in anaerobic shake flasks at a biomass concentration of 7.7 g L^{-1} with 4.5 mM acetate medium (Section 2.2.1).

In general it appeared that 1.2 Cmoles of glycogen were consumed per Cmole of acetate removed. This was a higher amount of glycogen consumption than that observed by other researchers [58, 69] of 0.50 moles glycogen per mole of acetate. The consumption of glycogen could be related to the disruption of the biofilm matrix making polysaccharide material more bioavailable for consumption than in the laboratory scale system where it was likely to be an integral part of the biofilm matrix [70, 71].

Small scale experiments to test the effect of biomass pretreatment on acetate uptake and storage

In contrast to the laboratory reactor trials, the preparation of biomass for the small scale tests involved rigorous treatment to remove excess PHB and to eliminate oxygen from the flask. An additional experiment was performed to mimic more closely the

conditions in the reactor (Section 2.2.3) to clarify the role of glycogen and oxygen in the biofilm.

The same pattern of acetate uptake, PHB production (1.4 Cmoles PHB produced per Cmole of acetate removed) and glycogen removal (2.3 Cmoles glycogen consumed per Cmole of acetate removed) was observed (Figure 2.13) as in the previous small scale tests (Figure 2.12). The actual ratios of glycogen and PHB metabolised per acetate consumed varied but not significantly (Figure 2.13) and the cause of the deviation was not clear. The similarity in behaviour of the small scale tests under all biomass pre-treatment conditions in terms of acetate uptake, indicated that the availability of oxygen to the shake flask had little impact on the behaviour of the system. It may be concluded that the low oxygen delivery (k_La = 1.1 h^{-1}) to the storage driven denitrification reactor did not substantially aid acetate uptake.

There was a smaller amount of PHB produced in the “reactor like” test (Section 2.2.3; exposure to oxygen during fill time) that may be a result of direct respiration of acetate (Figure 2.13). If the result is indicative of acetate oxidation, it suggests that the dominant role of oxygen in the storage driven denitrification biofilm was wastage of stored COD. It has been observed elsewhere that the supply of oxygen to the storage driven denitrification reactor has little effect on the ability of the culture to import acetate (Section 3.3.2).

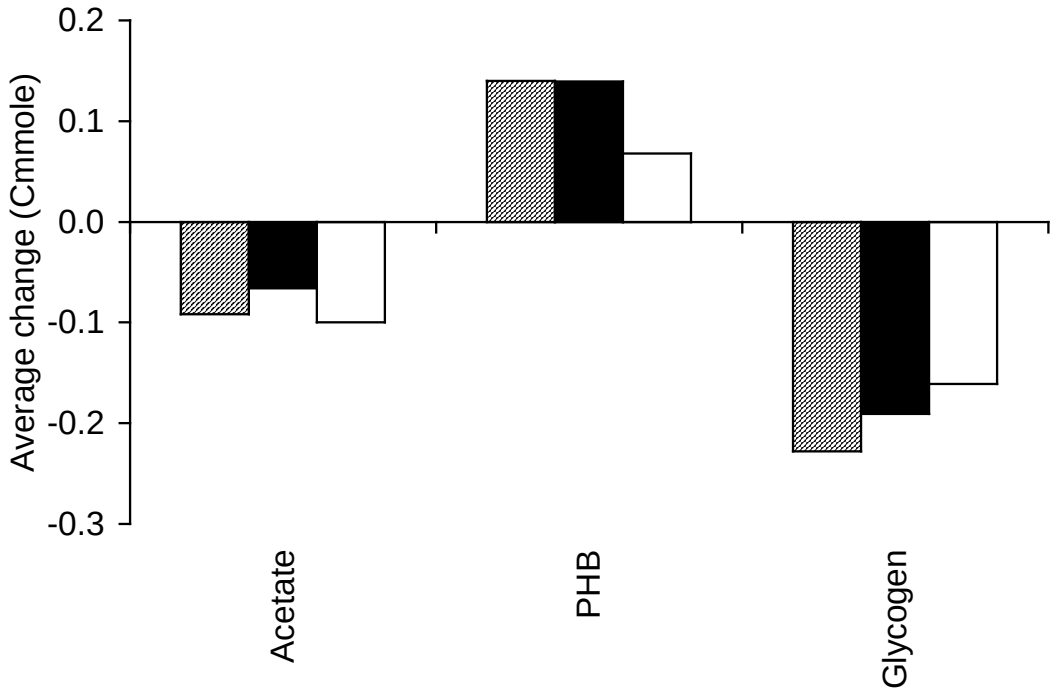

Figure 2.13 The effect of pre-treatment of the biomass on the carbon balance in the storage driven denitrification biomass in 20 mL medium in anaerobic shake flasks at a biomass concentration of 7.7 g L^{-1} with 2.6 mM acetate and 2.7 mM ammonia medium (Section 2.2.3); pre-treatment 1 (shaded columns), pre-treatment 2 (black columns) and pre-treatment 3 (white columns). Details of pre-treatments in Section 2.2.3.

2.3.4 Comparison of the Storage Driven Denitrification reactor with alternative literature systems

In principle, the sequestration of organic carbon by production of intracellular polymeric material will result in improved treatment performances for wastewater processes due to reduced loss of electron donor through direct oxygen mediated oxidation. While the level of carbon storage that the storage driven denitrification reactor exhibited was high, it remained to be evaluated whether this resulted in

improved nutrient removal performance compared to previously published studies on the stoichiometry of denitrification.

The storage driven denitrification reactor exhibited a lower rate of denitrification but compared very favourably in terms of C/N ratio of removal achieved with a range of published studies (Table 2.8). Abufayed and Schroeder [72, 73] studied denitrification kinetics with sludge as a primary carbon source for denitrification. The rate of denitrification observed was affected by the release rate of organic carbon. When nitrogen was not limiting, denitrification proceeded under zero order kinetics regardless of soluble or particulate carbon supply. With a six day sludge retention time, the denitrification sludge was able to achieve the complete removal of nitrogen with a carbon supply of 8.0 kg COD/kg N-NO_3^-. For those systems utilising natively available carbon either through predenitrification steps with raw wastewater or sludge as a carbon source or sequestered carbon, the rates were lower but generally required less input of external carbon [74].

Comparing the SDDN reactor rate performance with alternative systems can only be achieved with sludge specific rate data from all included studies. Since the amount of biomass contained in the SDDN can only be estimated, a thorough comparison of rates is not transparent and is included as a guide only.

About 40% of influent carbon of wastewater treatment processes is used for cell growth and maintenance [18, 19, 29]. This would result in minimum treatment ratios of around 4.7 kg COD/kg N-NO_3^- (Appendix A) but in practice, due to loss of COD by oxidation yields routine C/N ratios in the order of 7 – 8 kg COD/kg N-NO_3^- [18, 19, 29]. Without control of oxygen influx during the denitrification phase, the observed C/N treatment

ratio of the storage driven denitrification reactor was found to be 4.7 kg COD/kg N-NO_3^- (Section 2.3.3). It was estimated that a ratio of 3.6 kg COD/kg N-NO_3^- could be achieved if the supply of oxygen was excluded during denitrification. These ratios reduce the expected and routinely observed C/N treatment ratios in denitrification reactors as a direct consequence of the high level of storage of influent native carbon (Table 2.8). The storage driven denitrification sequencing batch biofilm reactor shows promise for a treatment system in combination with nitrification.

Table 2.8 Comparison of the storage driven denitrification reactor with other literature reactors/systems.

Reference	This study		Aesoy [74]			Beun [18]	Abufayed [72]	Jones [40]
	Figure 2.4	Figure 2.10	Hydrolysed sludge	Ethanol	Acetate			
Carbon requirement for nitrogen removal (kg COD/kg N-NO_3^-)	**4.25**	**4.69**	**8**	**4.5**	**NA**	**6.53**	**8.0**	**32.3**
Denitrification rate (mM NO_3^- /h)	**1.2**	**0.31**	**NA**	**7.4**	**6.0**	**3.5**	**NA**	**0.62**

2.3.5 Maximum storage capacity of reactor

It has been observed that very large amounts of PHB can be accumulated intracellularly with a theoretical maximum of 80% of the dry cell mass [36]. Previous research has indicated that stored PHB can routinely make up to 40 – 50% of the cell mass [75, 76] Applications of bacterially produced PHB come from several competing interests, including biodegradable thermoplastic production to carbon conservation for wastewater treatment. The minimal amount of PHB by dry cell mass observed in this

study suggests that considerably more could be stored if appropriate feed and conditions were imposed.

The storage driven denitrification biofilm batch reactor was given fresh synthetic wastewater and supplied, at a constant rate, additional acetate in order to increase the yield of PHB and to possibly observe the maximum storage yield of the biomass. For a period of 11 hours, the biomass was supplied with additional acetate at the rate of 3.7 mM h^{-1}. Over the course of the experiment, the soluble acetate concentration increased but at a slower rate than it was being added indicating that a proportion was being taken up by the biomass (Figure 2.14).

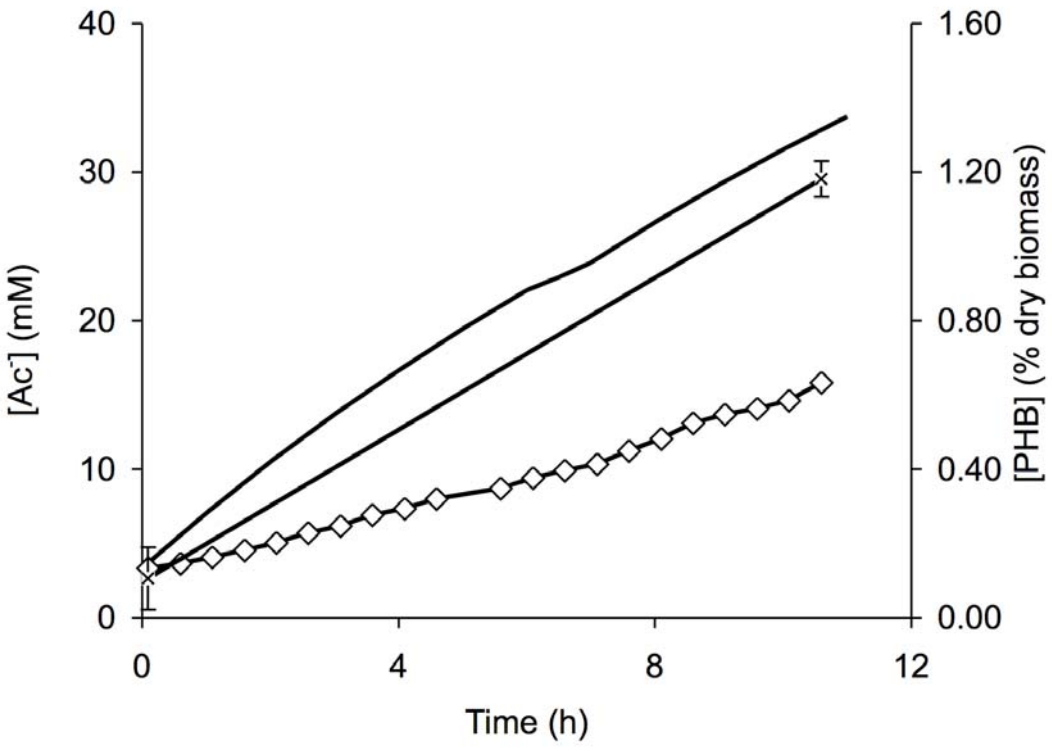

Figure 2.14 Effect of continuous addition of acetate to the storage driven denitrification reactor during the acetate uptake phase on acetate accumulation. Initial feed was 3.3 mM acetate and rate of addition of acetate 3.7 mM h^{-1}. Observed acetate in solution (◇), predicted acetate in solution (assuming no uptake of acetate; —) and PHB (✕). Error bars show standard error of PHB measurement.

Over the observed time period, 17 mM acetate was removed from solution and the PHB content of the biomass increased 11 fold (Figure 2.14). The PHB content of the biomass at the end of the experiment was still only 1.2% of dry cell mass (Appendix D). The storage driven denitrification reactor clearly had the ability to store considerably more COD than that routinely supplied in the influent wastewater although the actual maximum amount of COD that can be stored by the biomass was not observed.

2.4 Conclusions

A storage driven denitrification reactor was successfully established that could uptake acetate under strictly oxygen limited conditions and subsequently use the accumulated COD as an electron donor source for denitrification. The principal storage compound appeared to be PHB, which could be rapidly accumulated. Storage of up to 80% of the influent COD was observed, which could provide low carbon requirement for nitrogen removal of 3.6 kg COD/kg $N\text{-}NO_3^-$, a favourable result compared to published systems.

The energy source for acetate uptake was not clear although the results suggest that a mixed population of bacteria was selected for in which acetate uptake was powered by both polyphosphate degradation and aerobic oxidation of acetate. Nitrogen removal by the system was limited to less than 80% by the physical retention of ammonia during the acetate uptake phase. The combination of the storage driven denitrification biofilm sequencing batch reactor with a suitable nitrification culture needs to be assessed for full nutrient removal to be achieved.

Chapter 3. Characterisation of the storage driven denitrification reactor

Abstract

The previously described storage driven denitrification reactor, exhibiting good nutrient removal performance (carbon requirement for nitrogen removal down to 4.7 kg COD/kg N-NO_3^- and rate up to 3.0 mM NO_3^- h^{-1}) was further investigated to gain an understanding of the energy supply mechanism for COD uptake and storage. No clear single energy source was apparent for the storage driven denitrification biofilm reactor to facilitate acetate uptake and activation for storage. Results indicated that the removal of acetate was achieved by a combination of oxic and anoxic oxidation of PHB or acetate, anaerobic glycogen or poly phosphate metabolism for ATP production, due to the presence of a mixed population. While a high level of COD conservation through storage was achieved (up to 80% of influent acetate), a small improvement in additional conservation (30% reduction in carbon requirement for nitrogen removal) was achieved by the careful control of the acetate uptake phase length, reducing the wastage of stored COD under microaerobic conditions. The oxidation reduction potential (ORP) of the bulk liquor provided a reproducible signal coincident with complete acetate removal from the bulk liquor during the acetate uptake phase.

3.1 Introduction

A key limitation to nitrogen removal from municipal wastewaters is the supply of adequate electron donor for complete denitrification. Intracellular sequestration of COD

has been shown in this (Chapter 2) and other studies [18, 29, 37, 41] to reduce the amount of carbon required for nitrogen treatment due to the reduced waste from oxidation of external COD. Physical separation of COD removal from denitrification in the storage driven denitrification reactor encouraged the development of biomass that was capable of sequestering large amounts of influent acetate for use in denitrification (Chapter 2). As much as 80% of the acetate fed to the storage driven denitrification reactor was successfully stored by the biofilm and resulted in a C/N ratio of removal of 4.7 kg COD/kg N-NO_3^-. With improved oxygen control during the denitrification phase, this could be further improved to 3.6 kg COD/kg N-NO_3^- (Section 2.3.3).

The uptake of acetate for conversion to PHB requires 2 moles of ATP per mole of acetate [36, 77], which are used for the active transport of acetate and activation into acetyl CoA. Where respiration is used to produce ATP, assuming maximum efficiency of oxidative phosphorylation, 12 moles of ATP can be produced per mole of acetate oxidised. Thus, approximately 16.7% of influent acetate to an aerobic heterotrophic culture must be oxidised in order to produce sufficient ATP for transport and activation of the remaining acetate [37].

Previous results obtained from operation of the storage driven denitrification reactor indicated that only 11% of influent acetate supplied was oxidised (Section 2.3.3). Alternative sources of ATP production must therefore have been utilised by the storage driven denitrification biofilm in order to achieve the high level of acetate storage observed. Metabolic pathways for ATP production relevant to wastewater treatment systems include NO_x respiration, poly P hydroysis and degradation, and glycogen consumption. Phosphate release during acetate uptake was observed in the storage driven denitrification reactor (*eg* Figures 2.4, 2.5 and 2.10) and the presence of

glycogen was also observed. The possible contribution to the production of ATP by the storage driven denitrification reactor was not clear. The respiration of nitrogen oxides was not considered as the presence of nitrate and nitrite were reproducibly not observed during the acetate uptake phase of the storage driven denitrification reactor (*eg* Figures 2.4, 2.5 and 2.10).

The principal aim of this chapter was to determine the source(s) of ATP used in the uptake and intracellular accumulation of acetate by the storage driven denitrification reactor. In addition, an investigation into the possible impact of operational control parameters, such as liquor flow rate and phase length, on the performance of the storage driven denitrification reactor in terms of rate and carbon requirement for nitrogen removal was conducted in order to identify possible methods of improving of reactor performance.

3.2 Materials and Methods

3.2.1 Laboratory reactor design and operation

The reactor design used for work in this chapter was identical to that described in Chapter 2 (Section 2.2.2) with the exception of the trials where oxygen was strictly excluded from the reactor (Section 3.2.3). In general, the feed supplied to the reactor was the same as that used for the storage driven denitrification system (Section 2.2.1) unless otherwise specified in the results and discussion. Where changes were necessary, this was only in terms of the C/N ratio of the influent feed.

3.2.2 Analysis

All nitrogen and phosphorus compounds were measured spectrophotemetrically as described in Chapter 2 (Section 2.2.4). The acetate and PHB content of lyophilised biomass was measured by gas chromatography and glycogen content of lyophilised biomass was measured by either spectrophotometry or with a YSI glucose analyser (Section 2.2.4).

3.2.3 Exclusion of oxygen experiments

A set of experiments was performed in which oxygen was strictly excluded from entering the storage driven denitrification reactor (ie $k_La = 0\ h^{-1}$). To achieve this, the entire reactor (recycle vessel and column reactor) was degassed with nitrogen and sealed. A low permeability flexible nitrogen filled 'bladder' was connected to the reactor. The bladder allowed gas to move in and out of the reactor during the fill and decant phases for pressure equalisation. All feed solutions were degassed with nitrogen prior to use.

The integrity of the oxygen exclusion method was tested overnight during which the biomass was removed and deionised water was supplied as liquor. No oxygen was detected in the reactor liquor during the test period (Figure 3.1). A further modification was made in which a pump was connected to the recycle vessel that supplied a nitrate solution (6.2 mM) at a constant rate during the acetate uptake phase to provide an anoxic equivalent to the normal oxygen electron acceptor delivery conditions of the reactor (1mM $NO_3^-\ h^{-1}$). To test whether polyphosphate was a possible energy source

for ATP production, the storage driven denitrification reactor was fed a high phosphate (4.9 mM) nitrate feed during each denitrification phase in order to allow uptake of large amounts of phosphate for use in the subsequent acetate uptake phase.

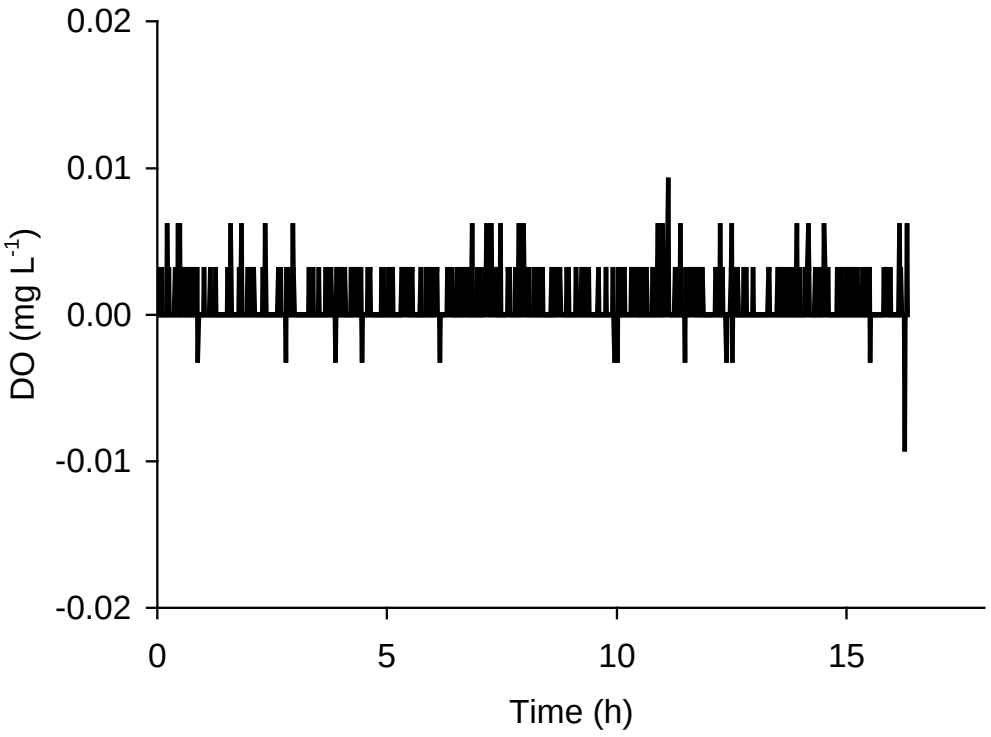

Figure 3.1 Testing of maintenance of negligible dissolved oxygen in the storage driven denitrification reactor when sealed and operated completely anaerobically (Section 3.2.3). The biomass chamber was filled with inert packing material, degassed with nitrogen and the reactor was fed deoxygenated deionised water. No biomass was present during the test. The liquor was circulated continuously for 16 hours to monitor any entry of oxygen.

In all tests, both phases of the storage driven denitrification reactor cycle were monitored in order to observe the removal and storage of acetate and the subsequent removal of nitrate due to acetate storage. In addition, a rinse phase of 5 minutes was included prior to each acetate uptake phase monitored to ensure that any residual nitrate or nitrite was removed from the biofilm.

3.2.4 Calculations

Measurement of k_La, calculation of OUR and hence oxygen consumed and C/N ratios of removal were performed as described previously (Section 2.2.6).

3.3 Results and Discussion

3.3.1 Investigation of ATP production in the storage driven denitrification reactor

The low oxygen supply (k_La = 1.1 h^{-1}; average DO = 0.4 mg L^{-1}; average OUR = 8.4 mg L^{-1} h^{-1}; Appendix B) to the storage driven denitrification reactor only provided approximately 66% of the expected ATP required for acetate uptake and storage. Additional respiration with nitrate or nitrite as electron acceptor would be excluded as these electron acceptors were not detected during the acetate uptake phase of the storage driven denitrification reactor. The possible sources of ATP production in the reactor were phosphate *via* polyphosphate accumulation and anaerobic glycogen consumption.

Polyphosphate can be used as a substitute for ATP when energy is required, releasing inorganic orthophosphate. The actual amount of phosphate released per mole of acetate uptake depends on the pH and varies between 0.42 mmole phosphate per mmole of acetate to 1.5 mmole phosphate per mmole of acetate [39, 78]. The average pH during the acetate uptake phase was approximately 7.6, which corresponds to a stoichiometric input of 0.55 mole phosphate released per mole of acetate taken up.

When polyphosphate is used as a source of ATP equivalents, release of orthophosphate is observed during COD uptake and uptake of orthophosphate during COD respiration [58]. Phosphate was observed to follow this pattern reproducibly during operation of the storage driven denitrification reactor (*eg* Figures 2.4, 2.5 and 2.10).

Two typical representative trials of the storage driven denitrification reactor were analysed for the respective contributions of phosphate release and oxidative respiration (Table 3.1). The combined ATP provision from phosphate release and oxidative phosphorylation was insufficient to wholly account for the uptake of acetate observed. The shortfall of ATP budget was at least 25% of that required (Table 3.1).

Table 3.1 Estimated contribution in the uptake of acetate by the storage driven denitrification biofilm from oxidation and phosphate release.

Feed C/N ratio (Influent acetate concentration)	**Contribution by oxygen**		**Contribution by phosphate**		**Estimated total acetate transported or oxidised**
	Oxygen consumed	Acetate oxidised (% acetate transported) [6]	Phosphate released	Estimated acetate uptake (% acetate transported)[7]	
3.9 kg COD/ kg N (1.8 mM) [8]	0.38 mM	0.19 mM (64%)	0.10 mM	0.18 mM (10%)	74 %
8.3 kg COD/ kg N (4.4 mM) [9]	0.67 mM	0.34 mM (46%)	0.21 mM	0.38 mM (8.6%)	55 %

Small scale tests (Section 2.2.3) with representative samples of biomass from the storage driven denitrification biofilm were conducted in order to determine the stoichiometric relationship between phosphate release and acetate uptake. No clear,

[6] Based on Third *et al* [37], 16.7% oxidation of influent acetate to uptake remaining acetate (Appendix B).

[7] Based on literature data [39, 78] where phosphate release depends on pH and falls in the range 0.42 – 1.5 mmol PO_4^{3-} per mmol of acetate. Average pH (7.6) during a typical acetate uptake phase used for analysis.

[8] Data obtained from the experiment shown in Figure 2.5.

[9] Data obtained from the experiment with nitrate feed in Table 2.5.

reproducible relationship between phosphate release and acetate uptake was observed (Figure 3.2).

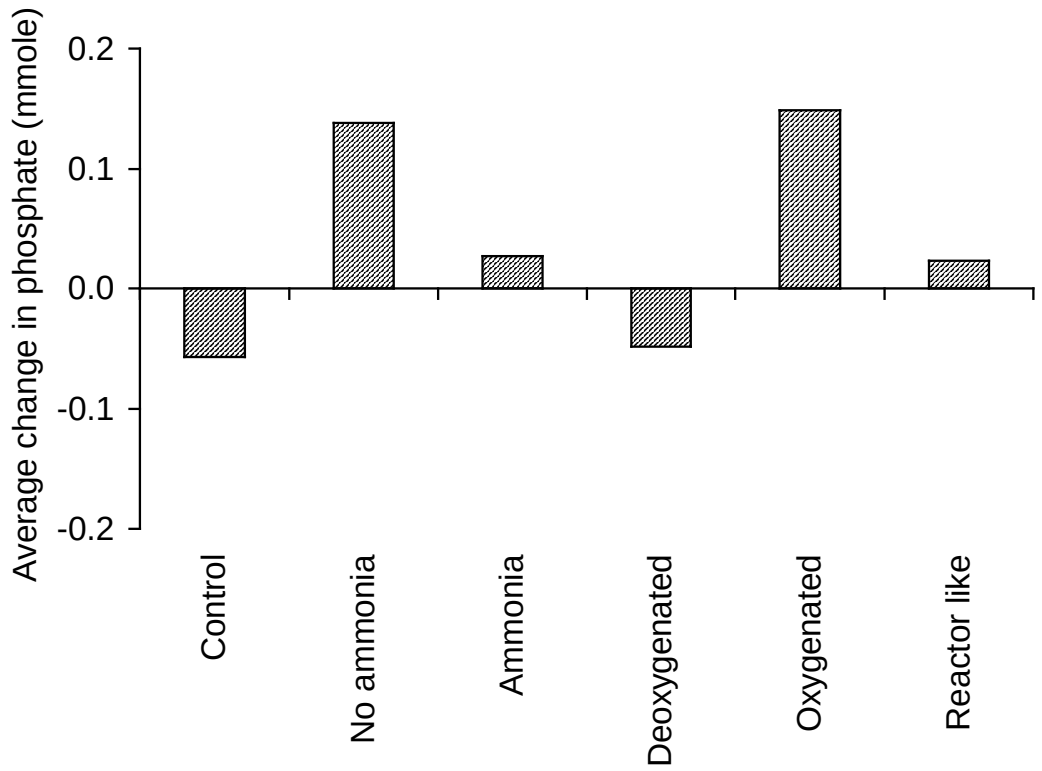

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Figure 3.2 Changes in soluble phosphate (mmole) in storage driven denitrification biomass suspended cultures removed from bioballs by washing and incubation (180 - 300 minutes) in a 20 mL anaerobic shake flask at a biomass load of 7.7 g/L with 3.7 acetate, 0.4 mM phosphate and, except in the no ammonia condition, 2.6 mM ammonia medium (Section 2.2.3) under a range of test conditions. Test conditions were: a) control in which no acetate, ammonia or phosphate fed; b) no ammonia in which biomass was given a medium as described in Section 2.2.3 without ammonia; c) biomass was incubated with the standard medium (Section 2.2.1); d) pre-treatment 1 (Section 2.2.3); e) pre-treatment 2 (Section 2.2.3) and f) pre-treatment 3 (Section 2.2.3).

While phosphate can be accumulated over successive cycles, there was no increased release of phosphate observed over successive cycles by the storage driven denitrification reactor during acetate uptake. This may indicate that a steady state population of PAOs had developed.

3.3.2 Dependence of acetate uptake on oxygen supply

While oxygen consumption contributed to the production of ATP during the acetate uptake phase, it was not the sole source of ATP production. A set of trials was conducted on the storage driven denitrification reactor under conditions that strictly excluded the supply of oxygen (Section 3.2.3). In each trial, an alternative substrate from which ATP may be generated was supplied (Section 3.2.3). The aim of these trials was to establish the oxygen dependence of acetate uptake by the storage driven denitrification biofilm.

The rate of acetate uptake was higher where oxygen or nitrate was supplied as an electron acceptor (Table 3.2), which supports the observation that anoxic or oxic uptake of acetate dominates (Table 3.1). A mixed process of ATP generation for acetate uptake is clearly evident however since acetate uptake still occurs where nitrate and oxygen were not supplied (Table 3.2).

The amount of phosphate released per acetate removed was higher under conditions where no electron acceptor was supplied but was still not sufficient to solely explain the uptake of acetate (Table 3.2). A third mechanism for ATP generation is therefore also supported by these results. The presence of a fermentable sugar, or sugar polymer such as glycogen, can be used to generate ATP *via* the conversion of glycogen to PHB [79]. The presence of glycogen and potential involvement in ATP generation was indicated previously (Section 2.3.3) and so the anaerobic degradation of glycogen by GAOs may be an additional source of ATP generation for acetate uptake.

Table 3.2 Acetate removal under oxygen limited supply conditions.

Alternative electron donor or energy source supply[10]	Feed [Acetate] [11]	Rate of removal Acetate	Release of [PO_4^{3-}]	% Acetate oxidised[12]	Carbon required for nitrogen removal (kg COD/kg N)
Normal (k_La = 1.1 h^{-1})	2.0	2.3	0.2	26	12
None	2.4	1.4	Not assessed	0	9.6
Nitrate	2.7	2.2	0.5	16	7.6
Phosphate	2.5	1.8	Not assessed	0	4.3

The uptake of acetate for conversion to PHB is an ATP dependent process [36, 77]. The generation of ATP in the storage driven denitrification biofilm appeared to be from a mixed metabolic process, possibly due to a mixed microorganism population and a plug flow like concentration gradient within the reactor [80], comprising the oxidative phosphorylation with oxygen or nitrate as electron acceptor, the accumulation and degradation of polyphosphate and fermentation of glycogen under anaerobic conditions. While the supply of oxygen results in a higher rate of acetate uptake, acetate uptake still occurred if oxygen, or a similar electron acceptor, was not supplied.

3.3.3 Conservation of carbon

Oxic respiration of acetate or PHB is the dominant route of ATP production in the storage driven denitrification reactor (Sections 3.3.1 and 3.3.2) but is also a significant source of wastage of stored COD. For example, 20% of the stored COD was wasted by the supply of oxygen during denitrification (Section 2.3.3). The operational length of

[10] Experimental condition to test either alternative electron acceptor (nitrate) or energy storage compound (phosphate from polyphosphate) for ATP production (Section 3.2.3)

[11] Concentrations given in mM; C/N ratios in kg COD/kg N-NO_3^- based on COD removed in acetate uptake phase and N removed in denitrification phase; Rate of removal in mM h^{-1}.

[12] Based on average OUR during the acetate uptake phase where k_La = 1.1 h^{-1} (Section 2.2.6) or nitrate supply and uptake during acetate uptake phase (Appendix B).

the acetate uptake phase may also contribute to COD waste by supplying more time and hence more oxygen than is necessary for acetate uptake.

If the continued delivery of oxygen due to continued liquor recycle caused a loss of PHB available for denitrification in the subsequent phase, then conservation of COD may be improved by ceasing the acetate uptake phase at or close to when acetate uptake is complete. To test this, the reactor times were adjusted so that the acetate uptake phase length was reduced to the average length of acetate uptake time observed in recent previous cycles (80 minutes *vs* 140 minutes).

Increased electron donor or reducing equivalents stored during acetate uptake phases were available during the subsequent denitrification phases than under the lengthier acetate uptake phase operation of the reactor (Table 3.3). The reduction by 60 minutes of the acetate uptake phase resulted in 43% less oxygen supplied and thus a potential saving of 7.8 mg L^{-1} stored COD. This was supported by the increased nitrate removal observed (0.3 mM more nitrate removed) compared with longer acetate uptake phase length trials.

Table 3.3 Performance of the storage driven denitrification reactor in successive cycles where acetate uptake was limited to 80 minutes.

Cycle #	Feed [Acetate] (mM)	Feed [NO_3^-] (mM)	N reduced (%)	Accumulation of NO_2^- (%)[13]	Rate of NO_3^- removal	Carbon required for nitrogen removal (kg COD/kg N)
1	3.2	1.5	72	23	1.4	13
2	2.6	1.6	74	20	1.5	10
3	2.6	1.7	81	13	1.5	9.0

[13] Accumulation of nitrite at end of denitrification phase expressed as a percentage of initial nitrate in denitrification phase feed.

It is possible that the improved removal of nitrate observed, particularly the increased rate of denitrification compared with normal trials (Table 3.3 and Figure 3.3) may have been due to adsorbed acetate in the biofilm. It has been previously demonstrated that substrates can be adsorbed from the bulk liquor into the biofilm matrix (Appendix E). As the rate of denitrification is known to be up to 2.5 times faster with a soluble exogenous electron donor such as acetate than with an intracellular polymeric source such as PHB [18, 26], the higher rate observed could be due to initial acetate availability in the denitrification phase.

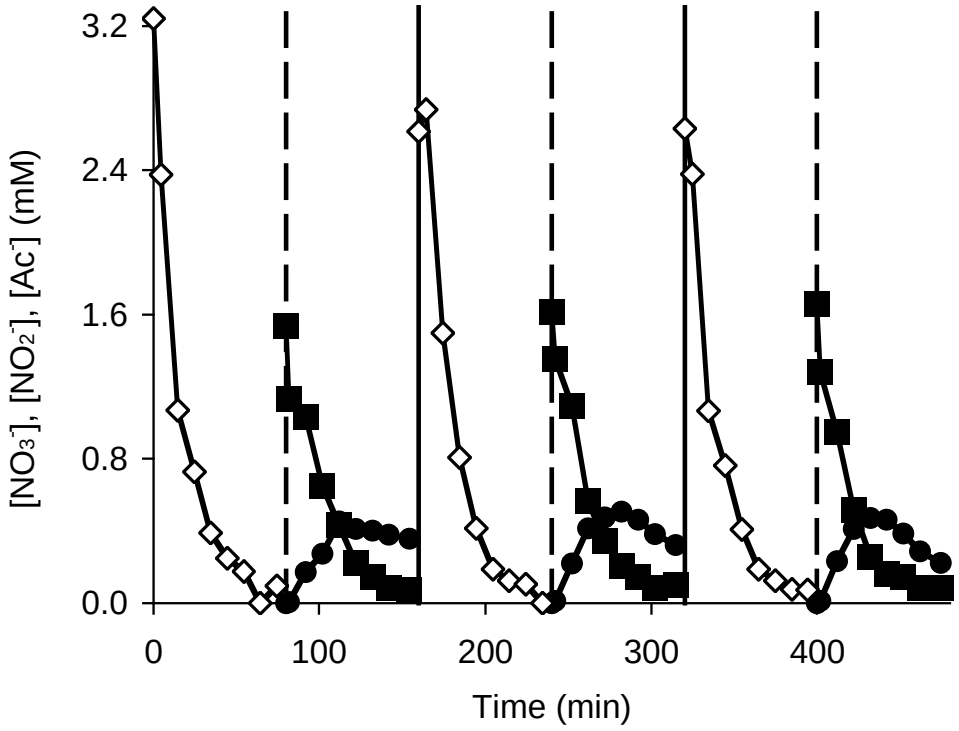

Figure 3.3 Effect of reduced acetate uptake phase length on storage driven denitrification reactor. (Feed solutions for each cycle shown in Table 3.3): Acetate (◇), nitrate (■) and nitrite (●). Vertical dashed lines indicate phase change from acetate uptake phase (80 minutes each) to denitrification phase (80 minutes each). Vertical lines indicate change in cycle (160 minutes each). Successive percentage of nitrogen removal in the three cycles observed was 72, 74 and 81%.

While it was not clear whether the cause of the improved C/N ratio of removal and denitrification rate was directly due to soluble acetate availability or decreased loss of PHB from limited oxygen exposure during the acetate uptake phase, there was a clear implication that the limitation of the length of the acetate uptake phase improved the performance of the storage driven denitrification reactor. Limitation of the acetate uptake phase length needs to be considered as an operational parameter to maintain the competitive performance of the storage driven denitrification system.

Possible signals for control of acetate uptake phase length

The storage driven denitrification reactor was controlled using static user defined time control. For possible improvement of performance of the storage driven denitrification reactor and the potential for development of a full scale reactor, it was necessary to determine if there were any surrogate indicators of suitable phase transition times such as redox potential, pH or dissolved oxygen, particularly for the acetate uptake phase given the possible savings of COD (above).

During the acetate uptake phase, the redox potential appeared to reach a local minimum that reproducibly coincided with the depletion of soluble acetate in the bulk liquor (Figure 3.4). To confirm the relationship between soluble external acetate exhaustion and the local minimum in ORP, a set of tests was conducted in which acetate, nitrate and phosphate were spiked into the recycle vessel 60 minutes into the acetate uptake phase.

The rapid reduction in ORP in response to the acetate spike indicated that the minimum of ORP was not directly related to acetate depletion (Figure 3.5). A clearer response,

however, was observed when phosphate was added (Figure 3.6). Although this meant there was no direct relationship between acetate concentration and ORP, the probable relationship between phosphate release and acetate uptake in the reactor means that the ORP could still be used as a surrogate for acetate uptake completion. This conclusion was further supported by the fact that a nitrate spike had no direct effect on the ORP in relation to the local minimum usually observed (Figure 3.7). Due to the reproducible pattern of acetate depletion and ORP, the ORP remains a valuable tool for the online control of the reactor, as has been observed by other researchers [81].

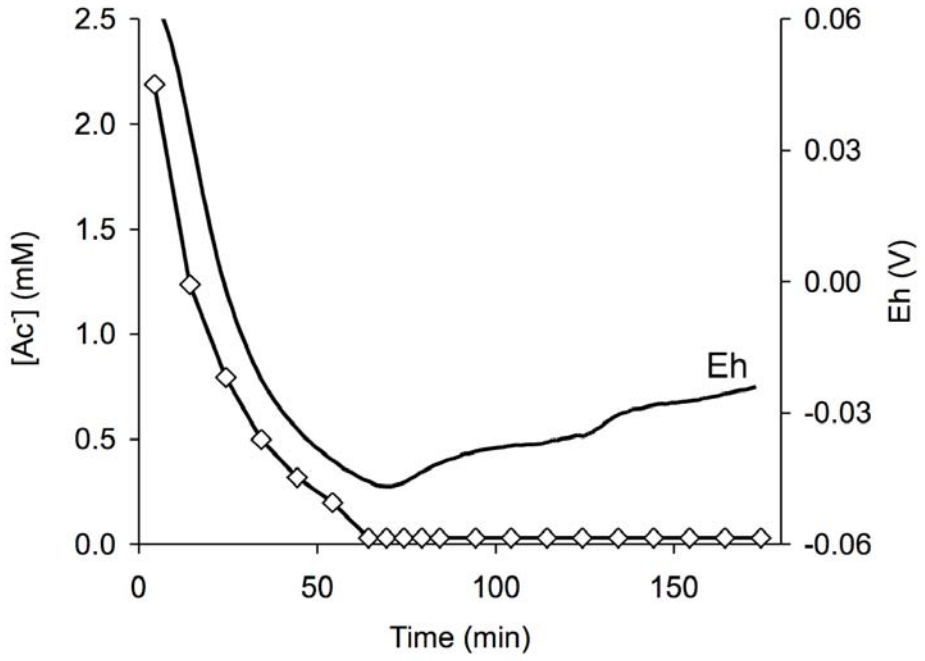

Figure 3.4 Redox potential and remaining acetate concentration during the acetate uptake phase of the storage driven denitrification reactor (Feed Acetate 2.2 mM): Acetate (◇). Acetate uptake phase length 180 minutes.

Under completely anoxic conditions, a reproducible bend, usually termed the 'nitrate knee' [81], in the ORP profile of a denitrification reactor can be used to obtain a feedback signal for control of cycle length. Under the microaerobic conditions employed in the storage driven denitrification reactor, the nitrate knee was not observed

(Figure 2.10). For control of the length of the denitrification phase, a NO_X probe would be more suitable.

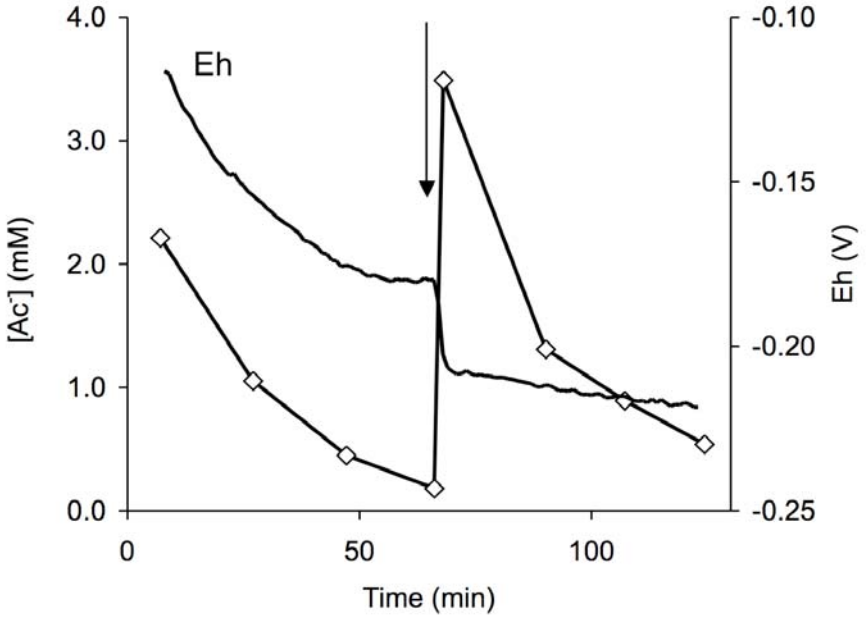

Figure 3.5 Redox potential and acetate concentration after the spike addition of acetate (added at 67 minutes; indicated by arrow) during the acetate uptake phase of the storage driven denitrification reactor. Feed Acetate 2.2 mM: Acetate (◇). Acetate uptake phase length 130 minutes.

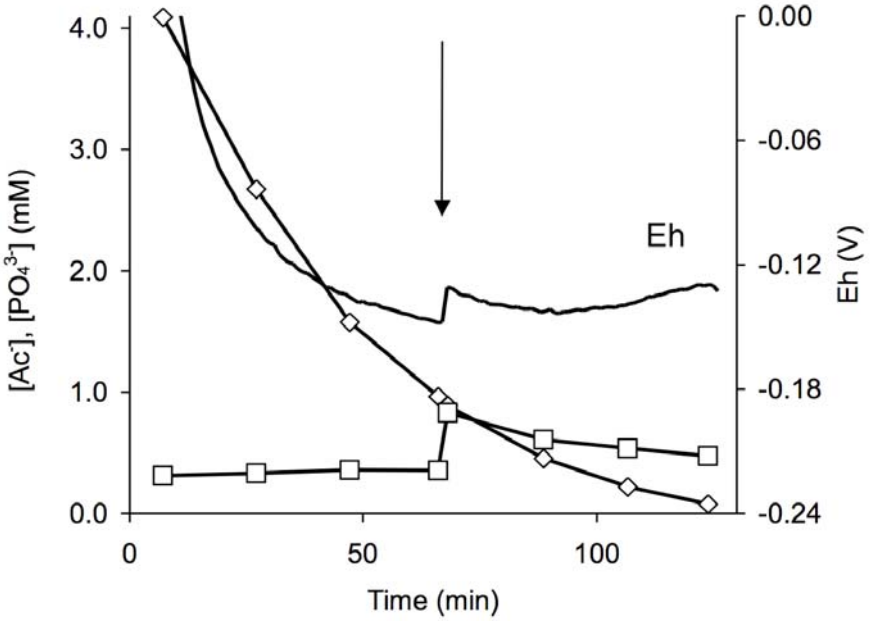

Figure 3.6 Redox potential and acetate concentration after a spike addition of phosphate (added at 67 minutes; indicated by arrow) during the acetate uptake phase of the storage driven denitrification reactor. Feed Acetate 4.1 mM: Acetate (◇) and phosphate (□). Acetate uptake phase length 130 minutes.

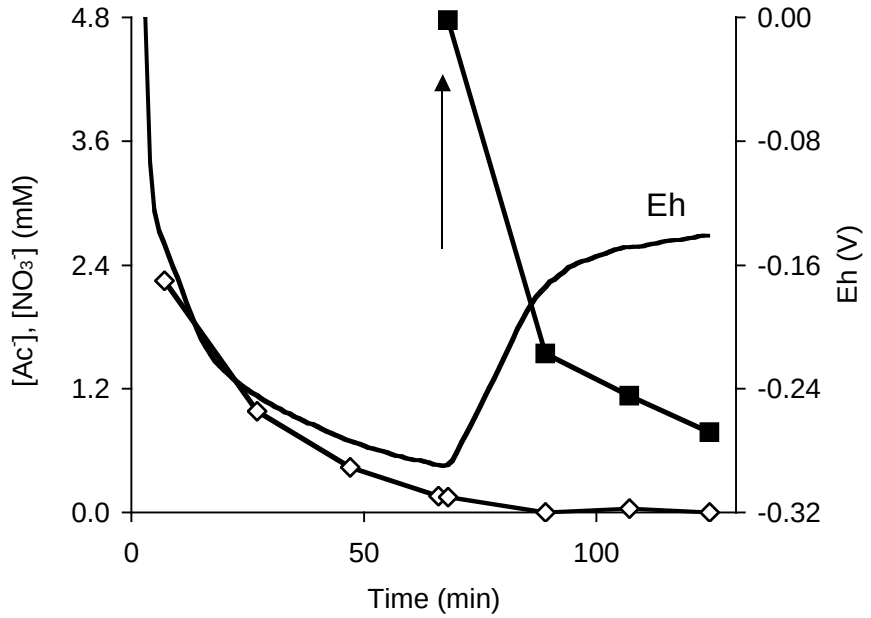

Figure 3.7 Redox potential and acetate concentration after a spike addition of nitrate (added at 67 minutes; indicated by arrow) during the acetate uptake phase of the storage driven denitrification reactor. Feed Acetate 2.2 mM: Acetate (◇) and nitrate (■). Acetate uptake phase length 130.

The concentration of dissolved oxygen in the bulk liquor generally increased when nitrate reduction was close to completion (*eg* Figure 2.5). However, since the supply of oxygen to this culture was minimal, this would not be a useful or reliable method of control. Like dissolved oxygen, pH was not deemed to be useful since the feed liquor was highly buffered (Section 4.3.3) and thus the expected increase in pH due to the reduction of nitrate was not clearly observed (*eg* Figure 2.5).

3.3.4 Effect of recycle speed on reaction rate

The operating cost of the storage driven denitrification reactor will include the cost of recirculating liquor through the system. While the actual relationship between power consumption and flow rate will specifically depend upon the efficiency of power use

and load on the pump motor, and hence the proportion of operating costs that pumping comprises, it is likely that operating costs associated with pumping could be reduced by reducing the operational flow rate [82]. The relationship between flow rate and reaction rates of the storage driven denitrification reactor should be established in order to identify a possible cost reduction of the process.

An increase in flow rate caused an increase in consumption rate of both acetate during the acetate uptake phase and nitrate during the denitrification phase by as much as 100% (Figure 3.8). Since the feed concentration (Table 3.4) in each phase was greater than the experimentally determined K_m for nitrate and acetate (Section 2.3.1), the enzymes involved in the rate determining step of each process should have been saturated and therefore the recycle flow rate should not have affected the reaction rates observed. As the reaction rate does increase with an increase in flow rate, it is clear that observation of the real V_{max} was obscured by some other effect.

Similarly to the apparent K_m measurements observed previously (Section 2.3.1), where the reactor was found to be diffusion limited due to the thickness of the biofilm, it appears that the observed V_{max} was found to be diffusion limited also. The observed acetate uptake rate was therefore the average observed reaction rate of each successive layer of the biofilm.

The nitrate reduction rate shows a similar dependence on liquor flow rate to the acetate uptake rate, however a maximum is reached above a flow rate of approximately 10 L h^{-1} (Figure 3.8). Although the same diffusion effect was involved, the magnitude differs

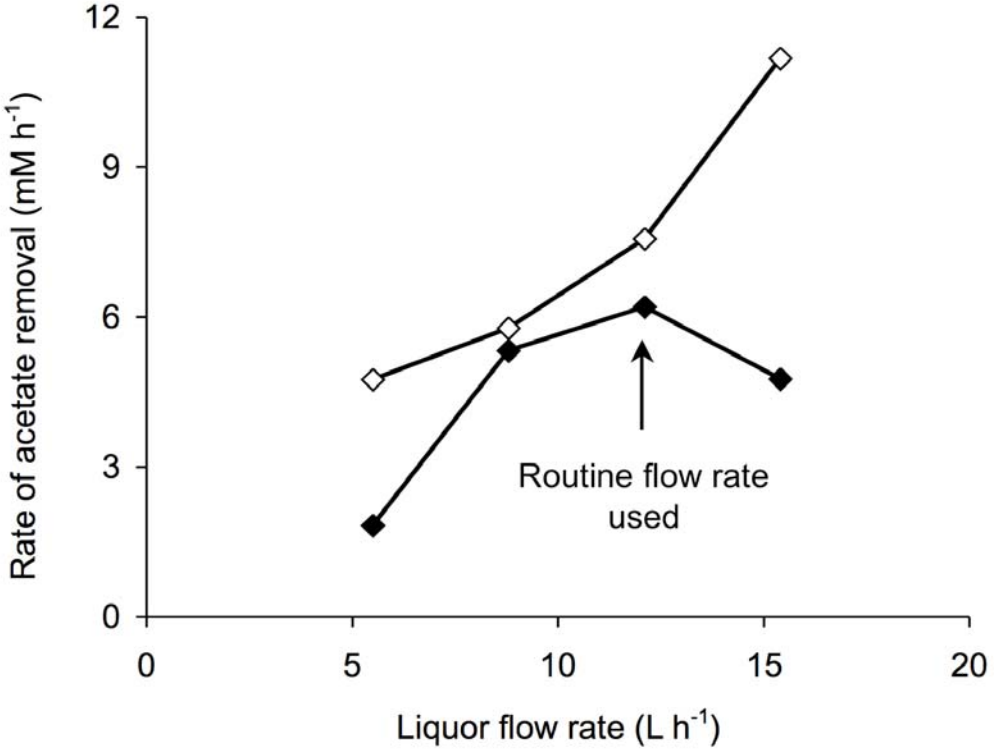

Figure 3.8 Effect of liquor flow rate on reaction rate in the storage driven denitrification reactor. Feed solutions given in Table 3.4. Acetate uptake phase length 60 minutes, denitrification phase length 300 minutes: Initial rate of acetate uptake (◇) and initial rate of nitrate uptake (◆).

Table 3.4 Effect of liquor recycle rate on storage driven denitrification performance.

Liquor flow rate ($L\ h^{-1}$)	**Feed**			**Accumulation of NO_2^- (%)[14]**	**Carbon requirement for nitrogen removal (kg COD/kg $N\text{-}NO_3^-$)**
	[Acetate] (mM)	[NO_3^-] (mM)	C/N (kg COD/kg $N\text{-}NO_3^-$)		
5.5	4.7	11.5	1.9	0.61	3.2
8.8	4.8	10.1	2.2	0.49	4.3
12.1	4.8	9.9	2.2	0.44	3.7
15.4	4.7	9.0	2.4	0.45	4.8

with nitrate as it has a higher diffusion coefficient than acetate (1.9×10^{-5} $cm^2\ s^{-1}$ *vs* 1.1×10^{-5} $cm^2\ s^{-1}$; stated at infinite dilution in aqueous solutions at 25°C [83]. Thus nitrate would diffuse at a faster rate than acetate causing more layers of the biofilm to be saturated with respect to nitrate. In addition, the reaction rate of denitrification may be limited, not by the supply of electron acceptor, but also by the supply of reducing power

[14] Accumulation of nitrite at end of denitrification phase expressed as a percentage of initial nitrate in denitrification phase feed.

(electron donor) such as PHB. Also, a higher liquor flow rate would increase the reactor k_La and hence oxygen delivery. At the highest flow rate tested (15 L h^{-1}), it is therefore possible that oxygen inhibition of denitrification occurred.

The dependence of reaction rates on liquor recirculation rate in the storage driven denitrification process is a clear reflection of the batch operation where the flow rate is analogous to stirring in a suspended culture reactor. The varying speed of flow achieved varying mixing performance and hence affected the diffusion within the biofilm. The relationship between flow rate and reaction rates in the storage driven denitrification reactor demonstrates a clear area for cost optimisation of the process where analysis of the actual performance of the pumps involved can be compared to the reaction rate to achieve a compromise between low cost and high performance.

3.4 Conclusions

The ATP required for acetate uptake and storage was produced *via* a mixed process involving some oxidative phosphorylation, some polyphosphate cleavage and some fermentation of glycogen stores. The exact proportion of each depended on the experimental conditions imposed. Increased conservation of COD was achieved by the control of the length of the acetate uptake phase. The reactor ORP provided a valuable tool for feedback control to signal the end of the acetate uptake phase. Since power cost for pumping is related to the speed of pumping utilised, a reduction in input of power could be achieved by lowering the flow rate with acceptable reduction in performance.

For complete nitrogen removal, the successful storage driven denitrification reactor will need to be combined with a suitable nitrification reactor for treatment of ammonia.

Chapter 4. Multistage wastewater treatment using separated storage driven denitrification and nitrification biofilms[15]

Abstract

The feasibility of combining a previously reported storage driven denitrification biofilm, where 80% of influent acetate could be converted to poly-β-hydroxybutyrate (PHB), with a suitable nitrification reactor, of either submerged or trickling filter design, to achieve complete biological nitrogen removal was tested. The reactor system exhibited complete biological nitrogen removal of waste streams with a C/N ratio as low as 3.9 kg COD/kg N-NH_3 at an overall nitrogen removal rate of up to 1.1 mmole NH_3 L^{-1} h^{-1}. While the efficiency and the rates of nitrogen removal were higher than those observed in simultaneous nitrification and denitrification (SND) systems, there were two problems that require further investigation: (a) the incomplete draining of the reactor causing ammonia retention and release in the final effluent, limiting the overall N-removal and (b) pH decreases in the nitrification step reducing the rate of nitrification if not corrected by appropriate pH adjustment or buffering.

4.1 Introduction

Biological nitrogen removal (BNR) is an important part of wastewater treatment and consists of two separate biological processes, nitrification and denitrification. Nitrification is performed under aerobic conditions by autotrophic bacteria that are slow

[15] The chapter has been published: [84]

growing and require long periods of time to reach sufficient biomass and activity level to significantly convert ammonia to nitrate [40]. Denitrification is performed by heterotrophs that require anoxic conditions and a source of electrons, usually chemical oxygen demand (COD), to convert the nitrate, produced by nitrifiers, into nitrogen gas [85]. It may be seen that these two processes have mutually exclusive requirements. During the air supply for nitrification, some COD will be oxidised [29, 86], limiting the efficiency of subsequent denitrification and increasing costs due to higher requirements for COD and oxygen supply.

To address the lack of carbon available for denitrification, recent developments in BNR technology, such as simultaneous nitrification denitrification (SND), have attempted to exploit the capability of wastewater sludge to store carbon sources under controlled conditions [29]. In these systems, the influent carbon is frequently stored as intracellular polymers, such as poly-β-hydroxybutyrate (PHB). The PHB can then be used instead of soluble COD as the principle carbon source for denitrification. Stored PHB has been shown to act as a slow release electron source for denitrification limiting its wastage through oxidation by oxygen [29, 40, 51]. This does not eliminate some of the difficulties in controlling the opposing oxygen requirements of the two nitrogen removal processes, nor the very different growth rates of the bacteria involved. A multistage system that separates the two microbial populations into different reactors has the advantage of allowing optimisation of, rather than compromising, each individual process.

Most multistage wastewater treatments systems developed to date rely on suspended culture sequencing batch reactor (SBR) technology [40, 48, 51, 87]. Carbon removal *via* intracellular storage as PHB occurs in an SBR and the subsequent high ammonia, low

carbon wastewater is then fed to a nitrification reactor where the ammonia is converted to nitrate. The effluent from the second reactor is then combined with the sludge from the initial SBR where the stored carbon is used for denitrification. Typical SBRs retain a high proportion, for example 60%, of liquor to maintain sufficient biomass levels after settling [37]. As a result, multistage systems that use SBR technology will retain a large proportion of the influent untreated ammonia from the initial feed source. To reduce this, Jones [51] operated with a 25% retained sludge blanket only. However, this still prevented 25% of the influent ammonia being untreated. In addition, the lower sludge blanket reduced reaction rates due to lower biomass levels [51]. A feasible alternative to SBRs, which may increase the amount of draining possible without affecting reaction rates, is the use of biofilm based reactors.

The aim of this paper was to investigate whether a previously described storage driven denitrification biofilm reactor, storing up to 80% carbon as PHB, could be combined with a suitable nitrification reactor to achieve BNR with minimal waste of oxygen and COD. Both sequencing batch reactor (SBR) and trickling filter (TF) based reactors for nitrification were tested.

4.2 Materials and Methods

4.2.1 Synthetic wastewater

In general, the feed supplied to the combined reactor operating in sequence was the same as that used for the acetate uptake phase of the storage driven denitrification

system (Section 2.2.1) unless otherwise specified in the results and discussion. Where changes were necessary, this was only in terms of the C/N ratio of the influent feed.

An additional feed was used to promote the development of an ammonia oxidising culture. The standard composition of the nitrification feed was (mg L^{-1}) NH_4Cl 160, KH_2PO_4 44, $NaHCO_3$ 125, $MgSO_4.7H_2O$ 25, $CaCl_2.2H_2O$ 300, $FeSO_4.7H_2O$ 6.25, and 1.25 mL L^{-1} of trace element solution (Section 2.1.1). This feed needed to be supplemented with additional buffering. Up to 2 g L^{-1} of $NaHCO_3$ was routinely used (Section 4.3.3).

4.2.2 Laboratory reactor design and operation

The storage driven denitrification sequencing batch biofilm reactor was operated precisely as described previously (Section 2.2.2). A nitrification reactor was connected to the storage driven denitrification biofilm *via* additional holding tanks to prevent mixing of the partially treated wastewater between stages. Full details of each reactor configuration are given below.

Nitrification sequencing batch reactor

As with the storage driven denitrification biofilm reactor, biomass was obtained from a local wastewater treatment plant and fed a selective medium to enrich for nitrification biomass (Section 4.2.1). A glass reactor with a 1 L operational volume (height 0.15 m, diameter 0.10 m) was fitted with a fermentor lid containing a turbine propeller stirrer, a feed inflow tube, an air line with attached bubble diffuser, a dissolved oxygen probe,

pH probe, oxidation-reduction potential (ORP) probe and an effluent withdrawal line (Figure 4.1). Air was supplied at a constant rate (24 L h^{-1}) by a needle valve mass-flow controller attached to the main airline. Stirring was maintained at 350 rpm. The k_La of this system was 45 ± 4.5 h^{-1} (Section 4.2.4). The average biomass concentration in the reactor was approximately 2.2 g L^{-1} and was kept in a steady state due to excess biomass washout as a function of SBR operation. A sludge blanket of 400 mL (40% of the total operational volume) was retained at the end of each cycle.

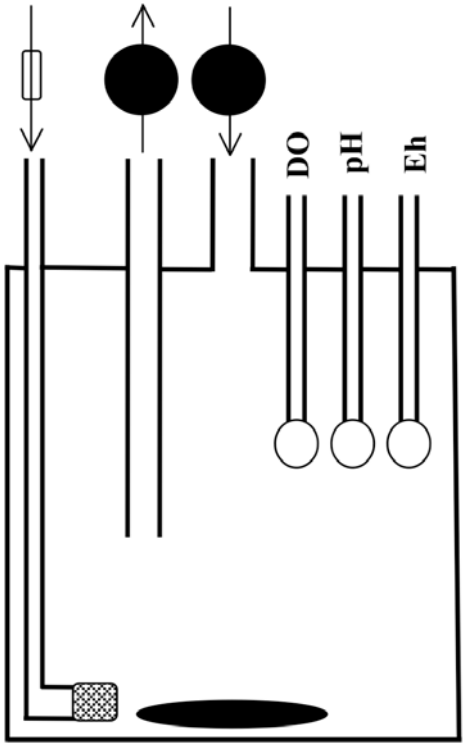

Figure 4.1 Nitrification suspended culture SBR. The reactor was operated in two phases, a react phase in which liquor was mixed and aerated and a settle phase, in which aeration and mixing was ceased in order to settle the biomass. At the end of each cycle, 60% of the operational volume was decanted and then refilled with fresh ammonia rich feed.

The SBR was operated in cycles of 180 minutes. The fill and decant phases were both rapid. Depending on the calibration of the pumps involved, they each generally made up no more than 4% of the total phase length. The length of settling phase was usually 10-

15% of the total phase length. A relatively short time was required due to the granular nature of the sludge (Section 4.3.1).

Nitrification biofilm sequencing batch reactor

To create a biofilm based nitrification reactor, the initial inoculum was taken from the granular biomass grown in the nitrification SBR. Additional loads of biomass for this reactor (to increase overall activity or reseed after storage) were taken from a medium scale (200 L) batch culture of nitrifiers with high activity [88]. Unlike the small scale SBR, the medium scale batch was fed a supplemented feed including yeast extract for trace element supply. As the object was to select exclusively for nitrifying biomass, yeast extract was not included in the small scale reactor feed as it afforded sufficient COD to support limited heterotrophic growth. The reactor was of identical design to that for the storage driven denitrification biomass and contained an equivalent number of bioballs for growth and support (Figure 4.2). This reactor was operated as an air filled trickling filter reactor to maximise oxygen transfer with minimal energy cost. To increase the surface area of contact between the reactor liquor and air to maximise oxygen transfer, a small reticulation sprinkler head was employed to disperse the liquor under peristaltic pump pressure.

Feed was batched to the recycle vessel and the reactor was operated in two phases. The first phase was a react phase in which liquor was recirculated through the trickling filter. The next phase was a drain phase in which pumping of the liquor was ceased and any accumulated liquor allowed to drain from the trickling filter chamber. The drain phase was analogous to the settle phase of the nitrification suspended growth SBR and similarly was routinely moderate in length (< 10% of 180 minute cycle).

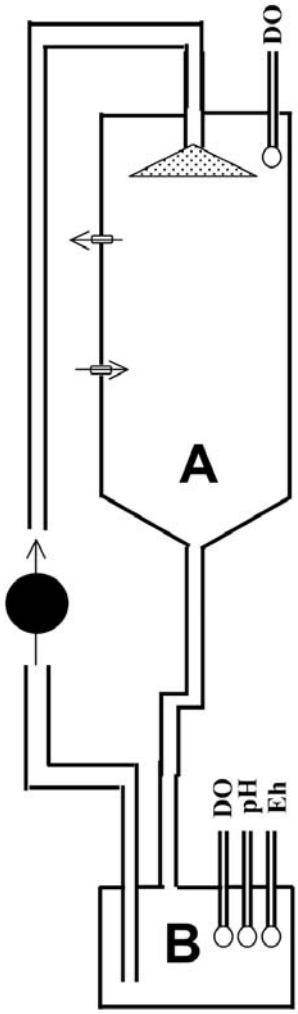

Figure 4.2 Nitrification trickling filter reactor with a biofilm chamber (A) and liquid recycle vessel (B). The reactor was operated in two phases, a react phase in which liquor was recycled through the trickling filter and a drain phase, in which the liquor was allowed to completely drain from the trickling filter. At the end of each cycle, the recycle vessel was drained and then refilled with fresh ammonia rich feed. The recycle pump is indicated by a solid circle with arrows for direction of flow.

As with the denitrification reactor, the recycle vessel was fitted with online probes for redox potential, pH and DO (Figure 4.2). A needle port was located in the upper air space of the column to relieve pressure. A second needle port was located in the lower half of the column reactor for air inlet. Addition of air was limited by a needle valve mass flow controller attached to the main airline, operated by computer control. The DO probe could also be located in the head space of the column reactor.

In each case, when the culture was inoculated, it took less than 24 hours for the bulk of the biomass introduced to attach to the biofilm. This may have been selected for based on the availability of both oxygen and ammonia within the biofilm chamber as opposed to the recycle vessel, where only ammonia was available in abundance. The cycle length of this reactor was identical to the SBR nitrification reactor.

Multistage multibiomass wastewater treatment system

For complete nitrogen removal using both the storage driven denitrification reactor and a nitrification reactor, the storage driven denitrification biofilm was combined, in series with each one of the two nitrification reactors described above. In this way, only a single feed was used in the system and the effluent from the initial acetate uptake phase as performed by the storage driven denitrification reactor produced the high ammonia, low COD feed for the nitrification SBR or TF which in turn produced the high nitrate, low COD feed for the storage driven denitrification reactor during the denitrification phase. As each of the reactors was operated in batch, it was necessary to have holding tanks between phases to collect and then redistribute the effluent from each reactor (Figure 4.3). The cycle times for each phase were as for the individual reactors involved.

4.2.3 Chemical analysis

Analysis of all compounds was performed as described previously (Section 2.2.4).

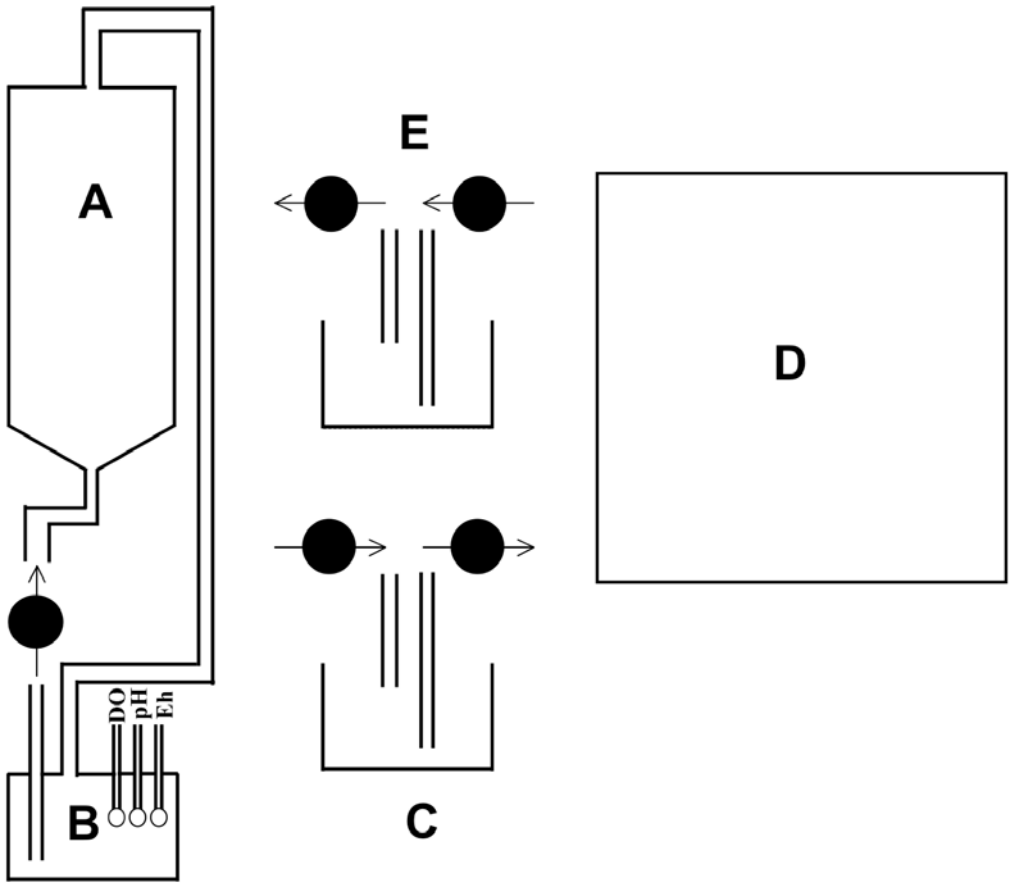

Figure 4.3 Operation of the multistage nitrogen removal sequencing batch reactor in three phases. Phase one: wastewater is fed to the storage driven denitrification biofilm reactor (A with recycle vessel B) for acetate uptake. Phase one is exactly analogous to the acetate uptake phase of the storage driven denitrification reactor (Section 2.2.2) with the exception that the effluent was transferred to a reservoir (C) rather than discarded. Phase two: the high ammonia, low COD effluent from Phase one (C) was fed to the nitrification reactor (D). The nitrification reactor was operated as either a suspended culture SBR (Figure 4.1) or a biofilm trickling filter (Figure 4.2). The nitrate rich effluent from the nitrification reactor was transferred to a reservoir (E). Phase three: the high nitrate effluent from Phase two (E) was transferred back to the storage driven denitrification reactor for denitrification. The effluent from Phase three was discarded.

4.2.4 Calculations

Overall nitrogen and ammonia removal rate

It was necessary to determine a transparent measure for the rate of nitrogen removal for the multistage reactor (Section 4.3.5). The overall rate of nitrogen and ammonia removal was determined by the total amount of nitrogen (or ammonia) removed per unit time based on the total cycle length including the acetate uptake phase.

Measurement of k_La, calculation of OUR and hence oxygen consumed for each individual reactor chamber and C/N ratios of removal were performed as described previously (Section 2.2.6). For measurement of the k_La of the trickling filter reactor, DO measurements were taken in the recycle vessel (Figure 4.2).

4.3 Results and Discussion

4.3.1 Development of nitrification reactors for multistage wastewater treatment

To achieve complete nitrogen removal, it was necessary to develop an independent nitrification reactor with optimised nitrification capacity. A suspended culture nitrification SBR was seeded with return activated sludge from a local wastewater treatment plant and supplied with a medium containing no COD (Section 4.2.1). The development of this culture with time was monitored and it was subsequently used to seed the TF reactor.

It had been reported previously [29, 39, 43, 89], and observed in this study, that the specific oxygen uptake rate (SOUR) observed in the nitrification reactor rapidly decreased when ammonia was depleted (Figure 4.4). As a result, the rate of change of the SOUR could be used as an indication of the exhaustion of ammonia supply, allowing to calculate the overall rate of ammonia oxidation. Over a succession of cycles, the biomass concentration in the SBR substantially reduced while ammonium oxidation rate increased (Figure 4.5). With prolonged exposure to a medium with no COD (Figure 4.5; cycle 8, 48 hours), the reactor reached a steady state in terms of biomass and nitrification rate. By this time, the biomass had become granular in appearance and settled rapidly. The duration of the SBR settling phase was reduced accordingly (Section 4.2.2).

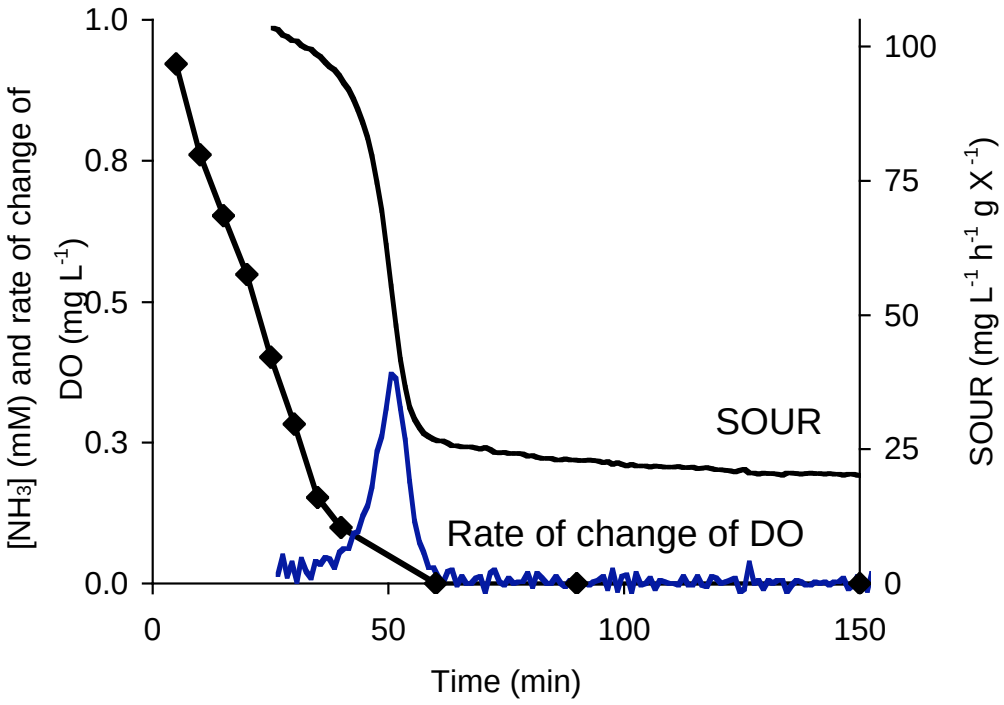

Figure 4.4 Ammonia loss during nitrification in a suspended culture SBR. Feed: Ammonia 0.9 mM. Ammonia (◆). Cycle length 150 minutes.

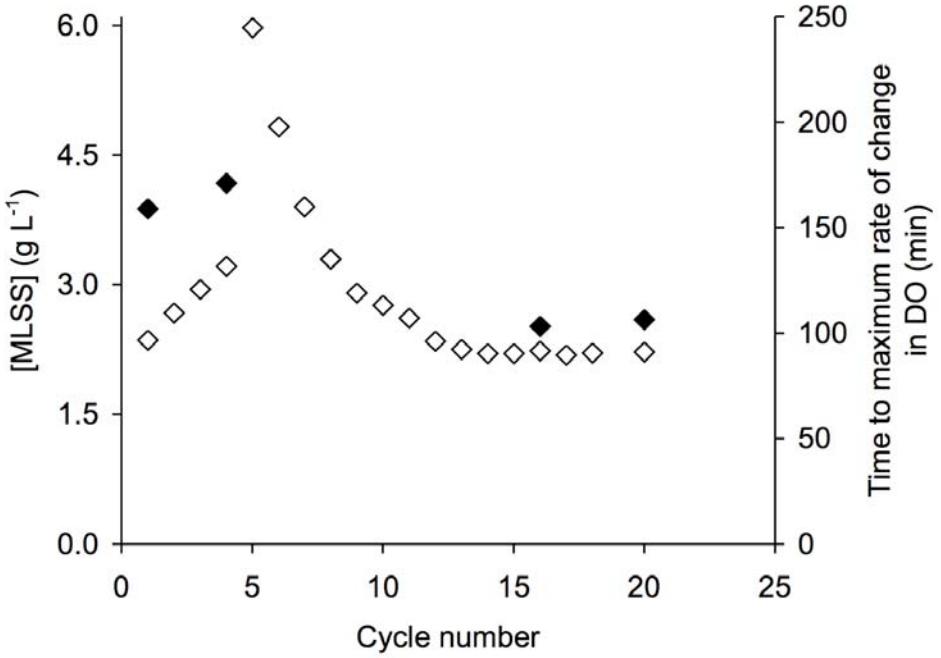

Figure 4.5 Change in time taken to reach maximum rate of change of DO (◇) and concentration of mixed liquor suspended solids (MLSS) (◆) in the nitrification suspended culture SBR from inoculation. Feed: Ammonia 3.0 mM. Cycle length 360 minutes.

Both ammonia and nitrite oxidisers were present in the reactor since nitrite was detected only in very low amounts throughout each cycle (Figure 4.6) and both *Nitrobacter* and *Nitrosomonas* species were detected *via* fluorescence *in situ* hybridisation (FISH) (Figure 4.7).

The granular biomass was used to seed the TF reactor once sufficient testing of the multistage system with the nitrification SBR was completed. This was done by repeated addition of granular nitrifying biomass in to the recycle vessel and waiting until it had attached onto the biofilm, which took less than 12 hours. As with the suspended culture reactor, minimal nitrite accumulation was observed (Figure 4.8) indicating that a substantial active population of both ammonia and nitrite oxidisers had developed.

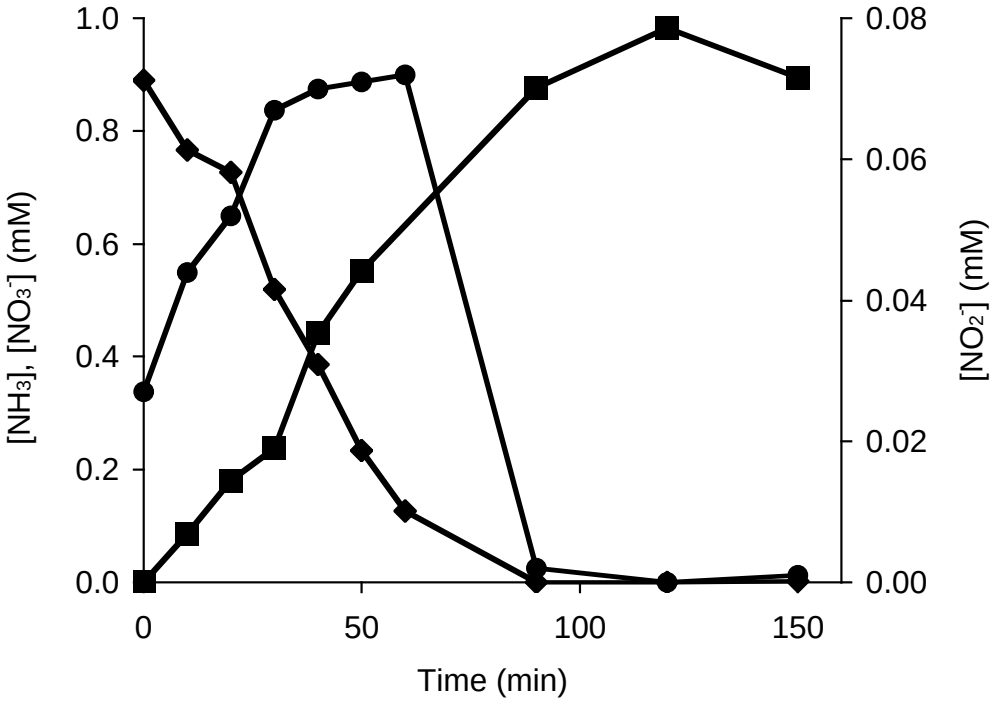

Figure 4.6 Accumulation of nitrite (●) and nitrate (■) from ammonia (◆) oxidation in the suspended culture SBR. Feed: Ammonia 0.90 mM. Cycle length 150 minutes.

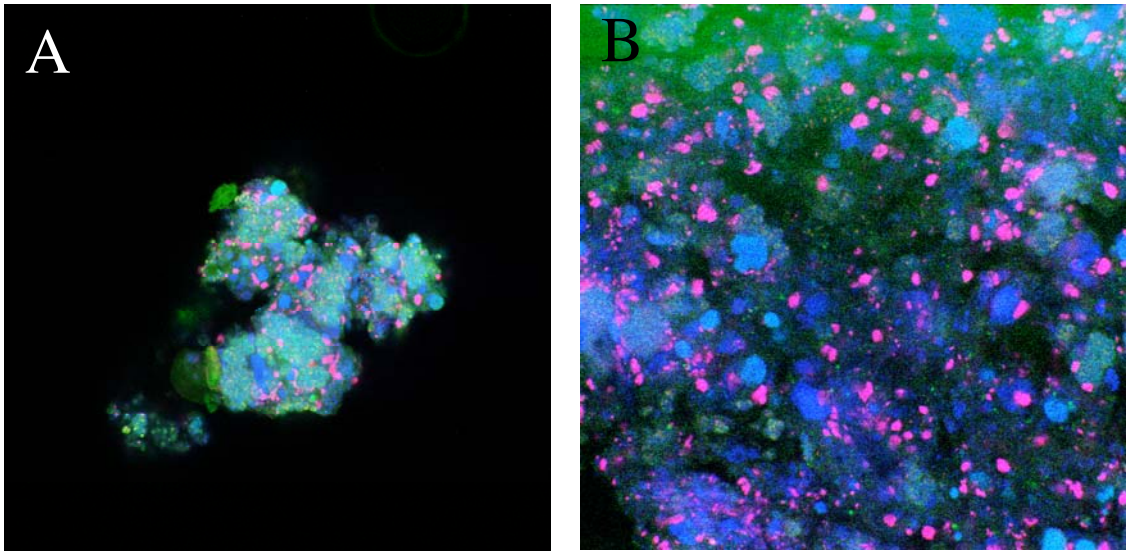

Figure 4.7 Fluorescence *in situ* hybridisation (FISH) of suspended culture SBR samples with NSP622+NIT for detection of *Nitrobacter* species (Cy3 labelled – blue); NSO1225 for detection of *Nitrosomonas* species (FITC labelled – green) and EUBMix for detection of bacteria (Cy5 labelled – red) [90]. Both images show the presence of *Nitrosomonas* and *Nitrobacter* species. Images courtesy of Brenton Gibbs.

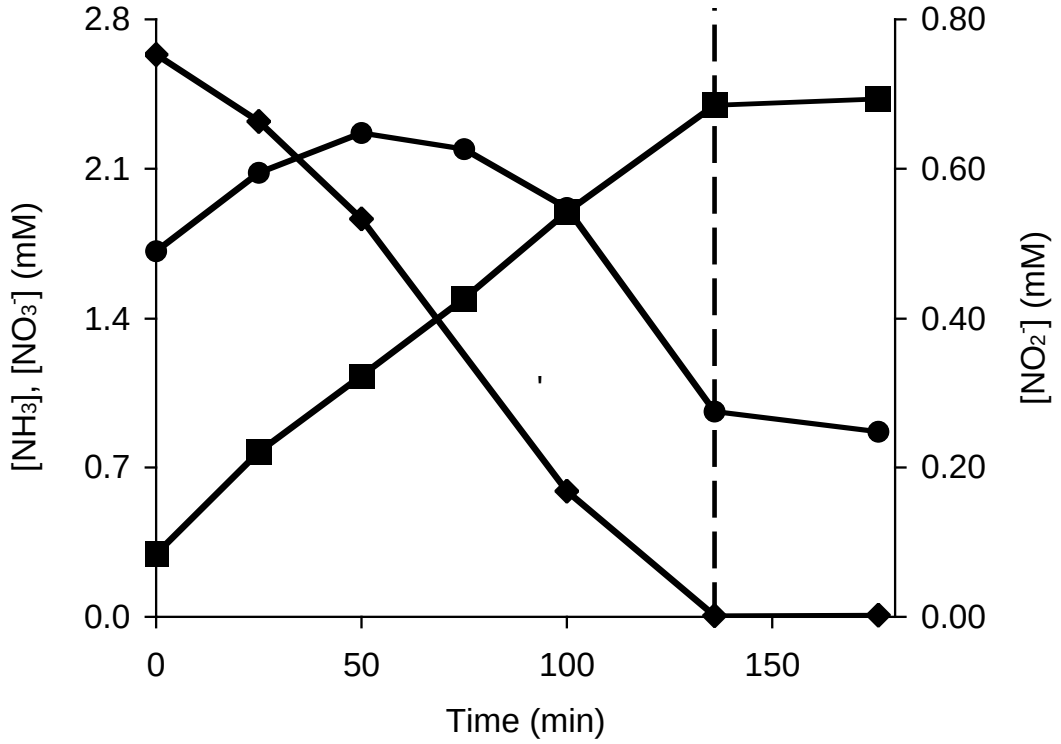

Figure 4.8 Accumulation of nitrite (●) and nitrate (■) from ammonia (◆) oxidation in the trickling filter biofilm nitrification reactor. Feed: Ammonia 6.6 mM. Vertical line indicates phase change from react phase (136 minutes) to drain phase (40 minutes). Reactor k_La 9.9 ± 1.9 h^{-1}.

Kinetics of ammonia and nitrite oxidation in the SBR and TF nitrification reactors

For both reactors, the nitrite and ammonia oxidation under substrate saturation improved with time (Table 4.1) indicating that the amount of active nitrifying biomass present in the biofilm had most likely increased. The half saturation constants, K_m, reflected those in literature (Table 4.1).

Table 4.1 Observed ammonia and nitrite oxidation in the TF and SBR reactors.

Nitrogen substrate	SBR		TF				Literature	
	K_M (R^2) (mM)	V_{max} (mM h^{-1})	Time 1		Time 1 + 1 month		K_M (mM)	
			K_M (R^2) (mM)	V_{max} (mM h^{-1})	K_M (R^2) (mM)	V_{max} (mM h^{-1})		
Ammonia	1.0 (0.96)	1.2	0.05 (0.98)	0.57	0.30 (0.98)	2.5	0.72 [7]	0.036 [91]
Nitrite	1.3 (0.996)	9.2	0.12 (0.98)	1.06	15.4 (0.9991)	82.6	1.29 [7]	1.6 [92]

Effect of recycle rate on ammonia removal rate in the TF nitrification reactor

As the oxidation of ammonia by nitrification microorganisms is an obligately aerobic process, changes in the supply of air, *via* manipulation of reactor k_La for example, may influence the rate of ammonia oxidation observed. Previous workers [39, 91, 92] have determined the oxygen half saturation constant for ammonia and nitrite oxidation.

Determination of the TF reactor k_La indicated that there was a direct relationship between the mass transfer coefficient and the flow rate of liquor in the reactor (Figure 4.9). As with a suspended culture SBR reactor, under oxygen limitation, a change in k_La of the TF reactor would be expected to influence the nitrification rate. In the TF reactor however, the ammonia removal rate was not substantially affected by the change in oxygen delivery (Figure 4.10).

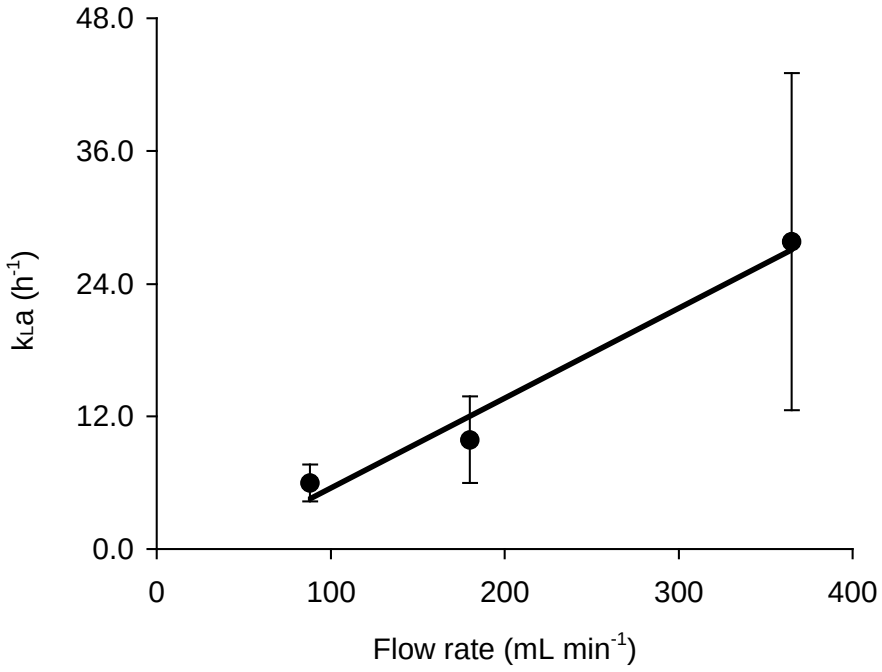

Figure 4.9 Effect of flow rate on the k_La of the nitrification trickling filter biofilm reactor. Error bars indicate 2 standard deviations around mean.

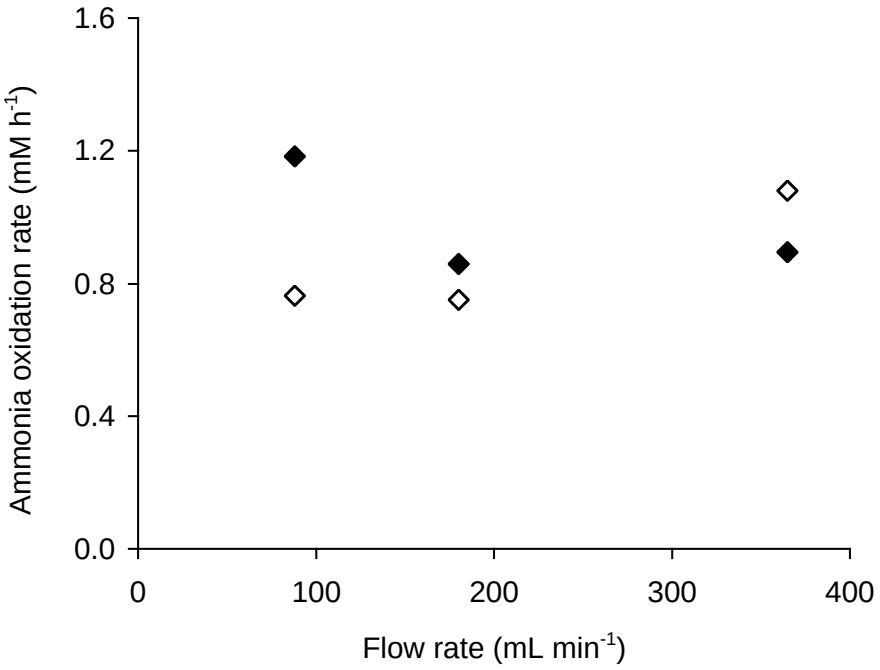

Figure 4.10 Effect of reactor k_La on the ammonia oxidation rate of the nitrification trickling filter biofilm reactor. Initial rate of ammonia oxidation (◆) and overall rate of ammonia oxidation (◇). Feed: Ammonia 2.5 mM. Cycle length 180 minutes (40 minutes drain).

The calculated amount of oxygen consumed, based on the recycle liquor DO and the reactor k_La (Section 2.2.6), appeared to be insufficient (< 50%) for the observed amount of conversion ammonia into either nitrite or nitrate (Equations 1.1 and 1.3). The results suggest that the biomass may have obtained the required oxygen directly from the gas phase in the head space of the TF reactor and that the dissolved oxygen concentration in the trickled liquid was not suitable as a basis for oxygen transfer calculations.

Effect of increased ammonia concentration on nitrification

The concentration of ammonia in the synthetic wastewater was comparable to that in real wastewater streams (Table 2.1). While this concentration represented a relevant level for municipal wastes, it did not inform on the ability of the TF nitrification reactor

to assist in the treatment of more concentrated waste streams such as piggery waste where ammonia can be as high as 500 mg L^{-1} [93].

The ammonia concentration in the nitrification reactor feed was doubled (from ~ 3 mM to ~ 6 mM) and the culture was monitored for 1 week to observe the effect that the increased concentration had on reaction rate. The overall rate of ammonia oxidation was not substantially altered under high ammonia loading compared to the normal feed (Figure 4.8; overall rate of ammonia oxidation with k_La = 28 h^{-1} with normal ammonia 1.2 mM h^{-1} compared to Figure 4.11; overall rate of ammonia oxidation with k_La = 28 h^{-1} with normal ammonia 1.1 mM h^{-1}). Of particular note was the near stoichiometric oxidation of ammonia to nitrite (Figure 4.11) rather than nitrate indicating that the population of nitrite oxidisers was almost completely inhibited as has been observed previously [94-96]. The loss of nitrite oxidation activity was not maintained long term. Once a lower ammonia feed was reintroduced, increased conversion to nitrate was observed after one week (Figure 4.12).

The inhibition of the nitrite oxidisers indicated that with high ammonia wastewaters, it may be possible to further reduce both the oxygen and COD requirement for nitrogen removal. A short circuit through nitrite, analogous to SND *via* nitrite processes [97, 98] may result in a 40% saving of COD and a 25% reduction in oxygen requirement (Figure 4.13). This would be expected to further improve the carbon requirement for the removal of nitrogen of the multistage system. A published separated sludge system with a nitrite short circuit achieves a very low C/N ratio of removal (2.7 kg COD/kg $N\text{-}NH_3$) as expected [99].

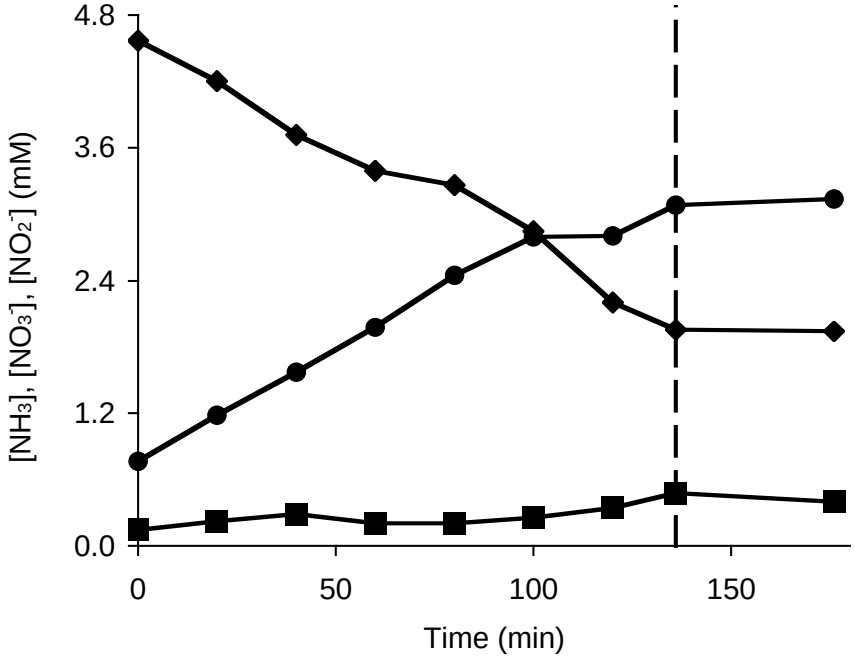

Figure 4.11 Effect of high ammonia concentration on the ammonia and nitrite oxidation of the nitrification trickling filter biofilm reactor. Feed: Ammonia 4.6 mM. Cycle length 180 minutes (40 minutes drain). Reactor k_La 28 ± 7.6 h^{-1}. Ammonia (◆), nitrate (■) and nitrate (●).

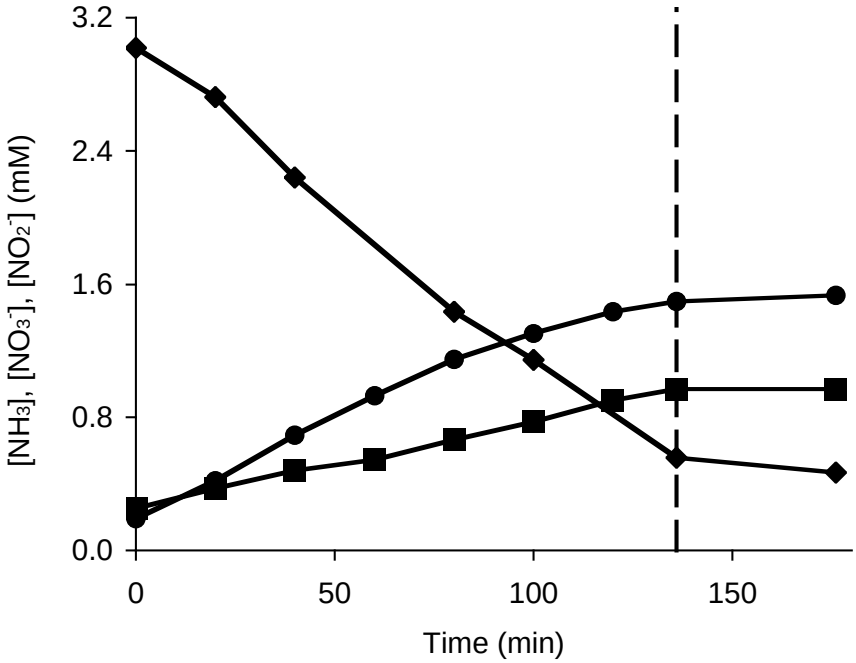

Figure 4.12 Recovery from high ammonia concentration on the ammonia and nitrite oxidation of the nitrification trickling filter biofilm reactor. Feed: Ammonia 3.0 mM. Cycle length 180 minutes (40 minutes drain). Reactor k_La 28 ± 7.6 h^{-1}. Ammonia (◆), nitrate (■) and nitrate (●).

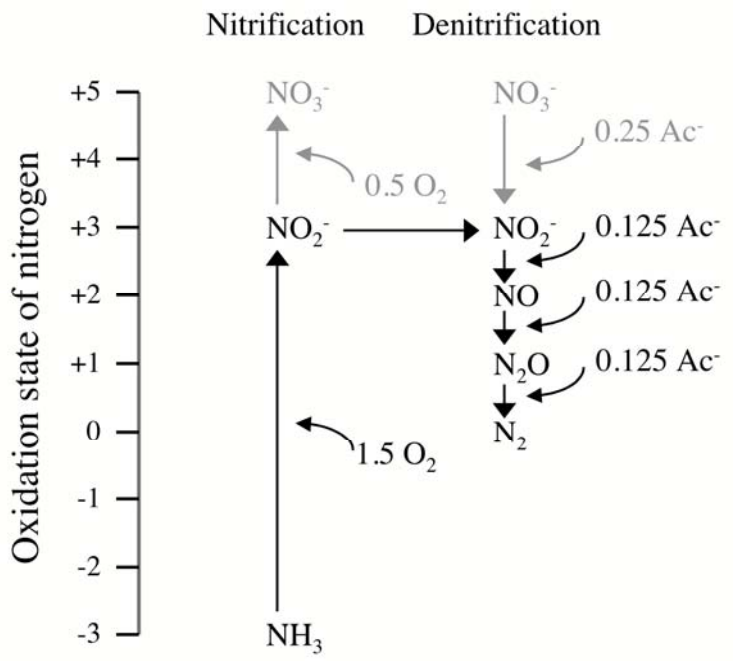

Figure 4.13 Theoretical short circuit between nitrification and denitrification providing savings of 40% COD (0.25 mol acetate/0.625 mol acetate) and 25% oxygen (0.5 mol oxygen/2 mol oxygen) as a result of nitrification-denitrification *via* nitrite instead of nitrate.

4.3.2 Can the storage driven denitrification reactor be successfully combined with a nitrification reactor for complete nitrogen removal?

The storage driven denitrification reactor achieved good nitrate reduction due to a high level of conservation of influent COD. Due to the high level of storage of COD exhibited by the storage driven denitrification culture (Section 2.3.3; up to 80%), routinely low C/N ratios of removal were achieved (down to 3.6 kg COD/kg N-NO_3^-).

The storage driven denitrification reactor was connected to two different nitrification reactors, a trickling filter (TF) and a suspended culture sequencing batch reactor (SBR) (Figure 4.3). Both removed substantial amounts of nitrogen using only the carbon stored from influent COD (Figure 4.14: 85% nitrogen removal with carbon requirement of 13

kg COD/kg N-NH_3; Figure 4.15: 81% nitrogen removal with carbon requirement of 8.0 kg COD/kg N-NH_3). The overall rate of nitrogen removal in the two examples was 0.44 and 1.1 mM h^{-1} respectively. The phosphate metabolism in the storage driven denitrification reactor was maintained regardless of connection with a nitrification reactor (Figure 4.14).

The rate limiting stage in both configurations was the nitrification phase. The rate of nitrification in both the TF and SBR systems was relatively low compared to the rate of denitrification. This may directly reflect the considerably larger biomass level in the heterotrophic biofilm compared to each of the autotrophic reactors. The rate of nitrification would be expected to be improved after building up higher levels of nitrifiers.

Substantially lower C/N ratios of removal were observed than those shown above (Figure 4.16) indicating that the nitrogen removal performance of the storage driven denitrification reactor was not substantially altered by connection to an external nitrification reactor. Approximately 10 - 20% of ammonia was still retained by the storage driven denitrification biofilm during acetate uptake (Figures 4.14, 4.15 and 4.16). Although each phase performed 100% removal of the targeted substrate (*eg* ammonia in the nitrification reactor), the physical retention of ammonia limited the overall nitrogen removal of the system. While a biofilm system is ideally expected to drain completely, in practice, the amount of drainage will depend on the amount of biomass present. According to adsorption modelling (Appendix E) of the SDDN biofilm, it is possible that up to 28% of liquor is retained in the biofilm. If all the liquid that is retained contains ammonia, then up to 28% of influent ammonia will be retained. The physical retention of ammonia has been observed in other biofilm systems [53].

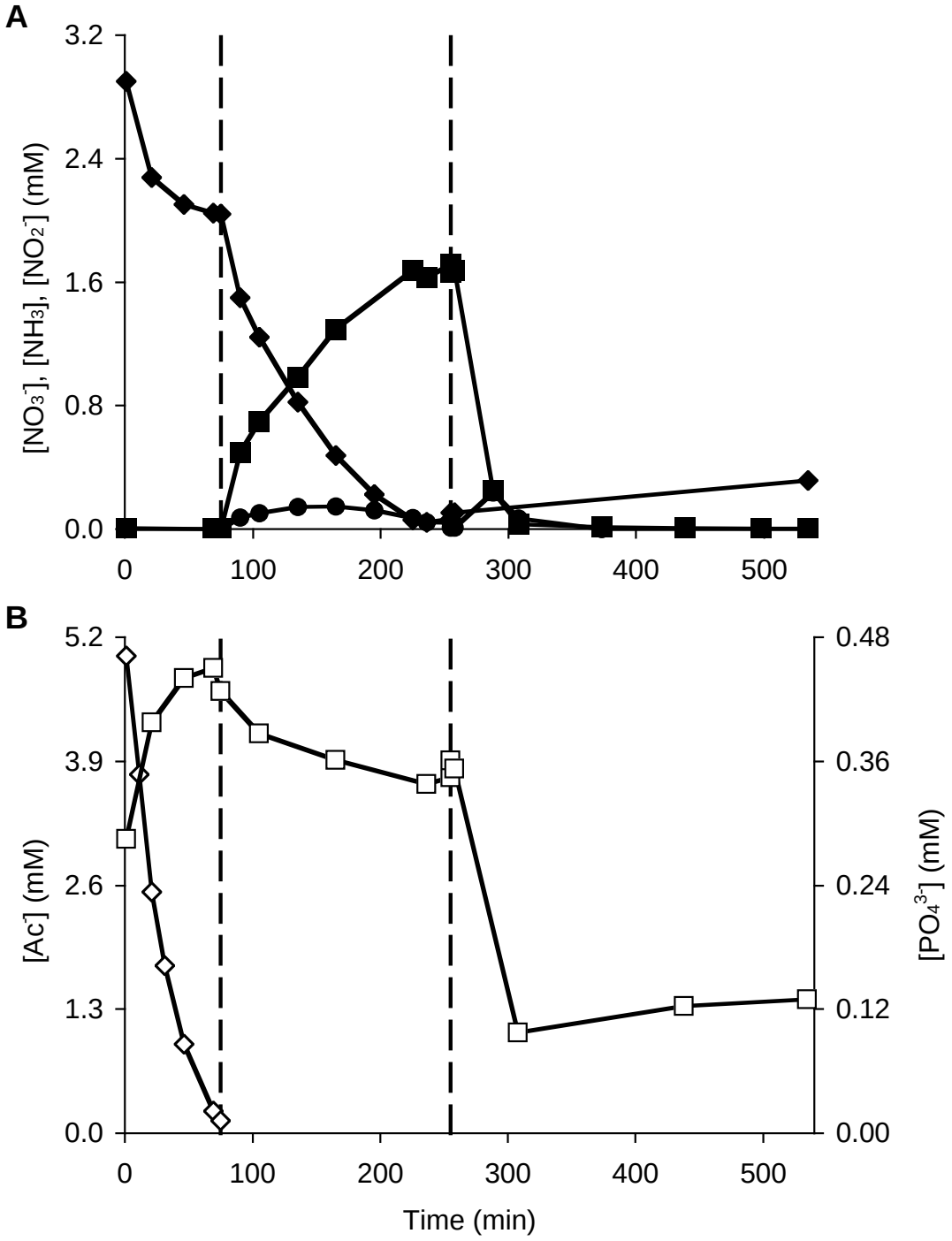

Figure 4.14 Typically observed metabolite behaviour during 3 stage nitrogen removal (Section 4.2.2). Stage 1 was acetate uptake in a storage driven denitrification biofilm reactor for acetate conversion to PHB from a synthetic wastewater (Section 2.2.1; Ammonia 2.9 mM and Acetate 5.0 mM; 7.9 kg COD/kg N-NH_3) Stage 2 was nitrification in a biofilm trickling filter reactor for ammonia conversion to nitrate. Stage 3 was denitrification in the storage driven denitrification reactor using stored PHB from Stage 1 to convert nitrate to nitrogen gas. A: Ammonia (◆), nitrate (■) and nitrite (●). B: Acetate (◇), phosphate (□). Vertical line indicates phase changes from acetate uptake phase to nitrification phase (75 minutes) and from nitrification to denitrification phase (255 minutes).

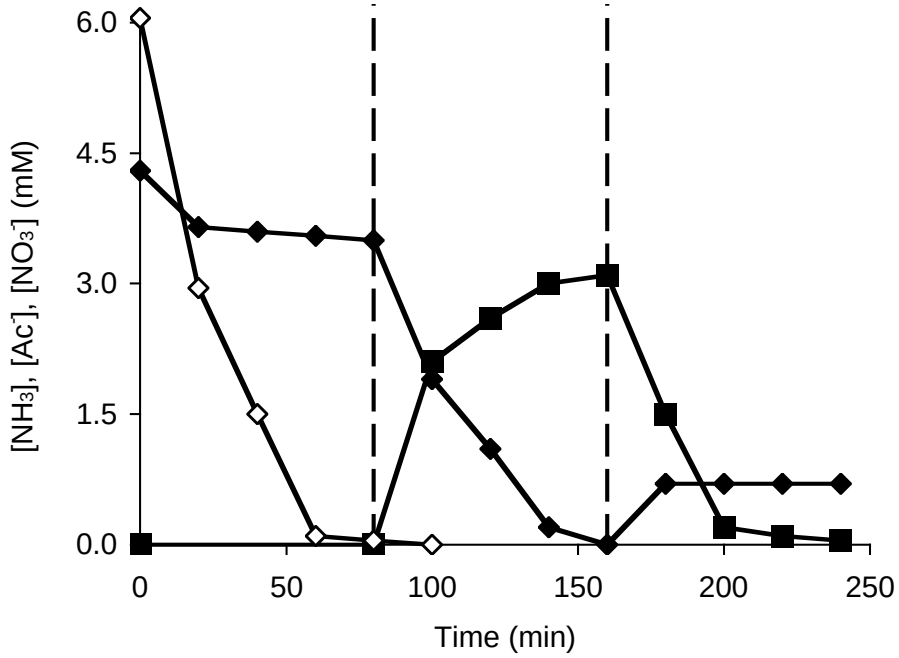

Figure 4.15 Typically observed metabolite behaviour during 3 stage nitrogen removal (Section 4.2.2). Stage 1 was acetate uptake in a storage driven denitrification biofilm reactor for acetate conversion to PHB from a synthetic wastewater (Section 2.2.1; Ammonia 4.3 mM and Acetate 6.1 mM; 6.4 kg COD/kg N-NH_3) Stage 2 was nitrification in a suspended culture SBR for ammonia conversion to nitrate. Stage 3 was denitrification in the storage driven denitrification reactor using stored PHB from Stage 1 to convert nitrate to nitrogen gas. Ammonia (◆), nitrate (■) and acetate (◇). Vertical line indicates phase changes from acetate uptake phase to nitrification phase (80 minutes) and from nitrification to denitrification phase (160 minutes).

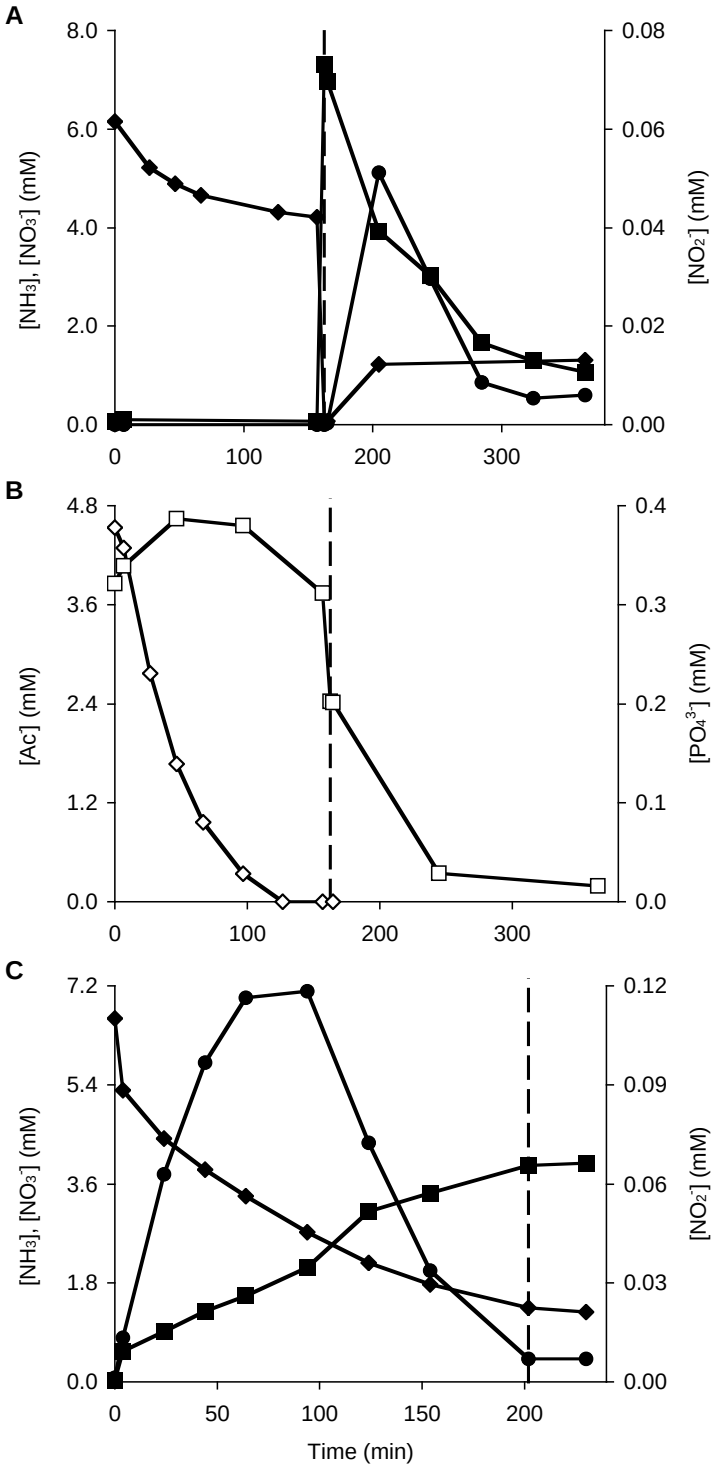

Figure 4.16 Removal of nitrogen from a low COD/N ratio synthetic wastewater by the multistage reactor with nitrification in a trickling filter biofilm reactor (Section 4.2.2). Stages 1 (acetate uptake) and 3 (denitrification) shown in A and B. Stage 2 (nitrification) shown in C. Feed: Ammonia 6.2 mM and Acetate 4.5 mM; 3.4 kg COD/kg N-NH_3. Ammonia (◆), nitrate (■), nitrite (●), acetate (◇) and phosphate (□). Vertical line indicates phase changes. In A and B: from acetate uptake phase to denitrification phase (163 minutes) and C: from react to settle in the nitrification phase (202 minutes).

4.3.3 pH drift in the separated nitrification reactors

The results of the multistage and nitrification reactor operation were obtained at a high level of buffering (between 0.5 g L^{-1} and 2 g L^{-1} $NaHCO_3$). Nitrifiers acidify their environment and are inhibited by low pH and therefore the separated activity of the nitrifiers results in a detrimental pH shift lowering the rate of nitrification [92, 100].

In successive cycles of the nitrification SBR, protons accumulated in the medium (Figure 4.17). The rate of pH drift observed was as much as –0.9 pH units per hour. With each depression in pH, the specific activity of the nitrifiers was lowered as would be expected. The use of a biofilm for nitrification was not expected to improve the accumulation of protons as the biofilms had been shown, in this study (Appendix D) to retain solute species. The pH of successive cycles in the TF nitrification reactor also showed an accumulation in effect (Figure 4.18). In addition, studies using microelectrodes to probe chemical gradients in biofilms have shown that the interior of the biofilm can be considerably lower in pH than the bulk liquor [13].

Buffer requirement for the nitrification reactors

The drift in pH during nitrification, either in the trickling filter or SBR configurations, inhibited nitrification activity. This limited nutrient removal in the multistage reactor as it not only resulted in low ammonia treatment it also prevented the supply of nitrate or nitrite to the storage driven denitrification biofilm. The synthetic wastewater was found to have an alkalinity of 770 ± 17 mg L^{-1}, which is considerably more than that observed in real wastewaters (Table 2.1). The impact of pH drift from nitrification of a real wastewater is therefore likely to be larger.

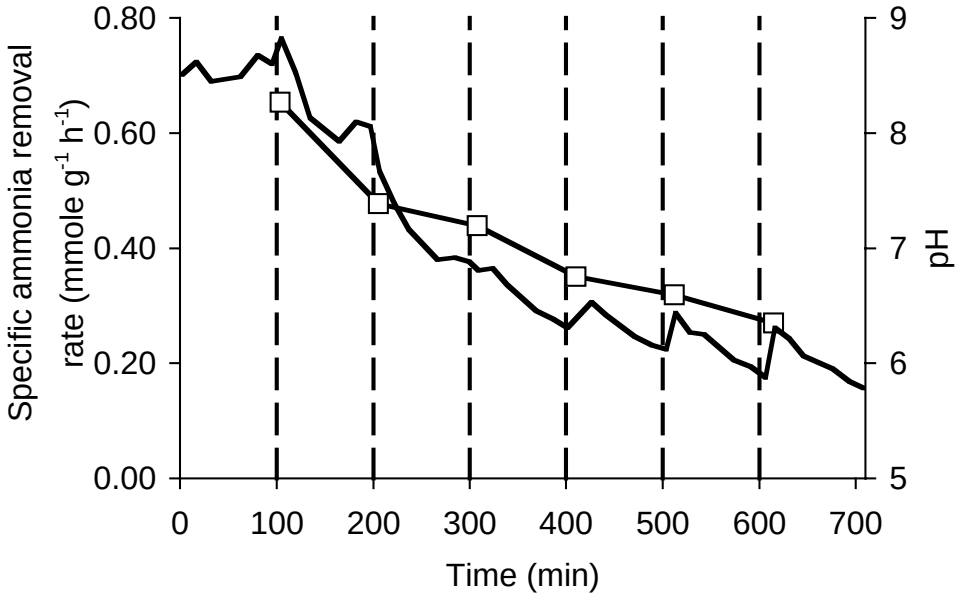

Figure 4.17 Effect of pH reduction in successive cycles of the nitrification suspended culture SBR on the specific ammonia oxidation rate (□). Feed: Ammonia 2.0 mM. Cycle length 100 minutes (15 minutes drain). Vertical lines indicate end of each cycle.

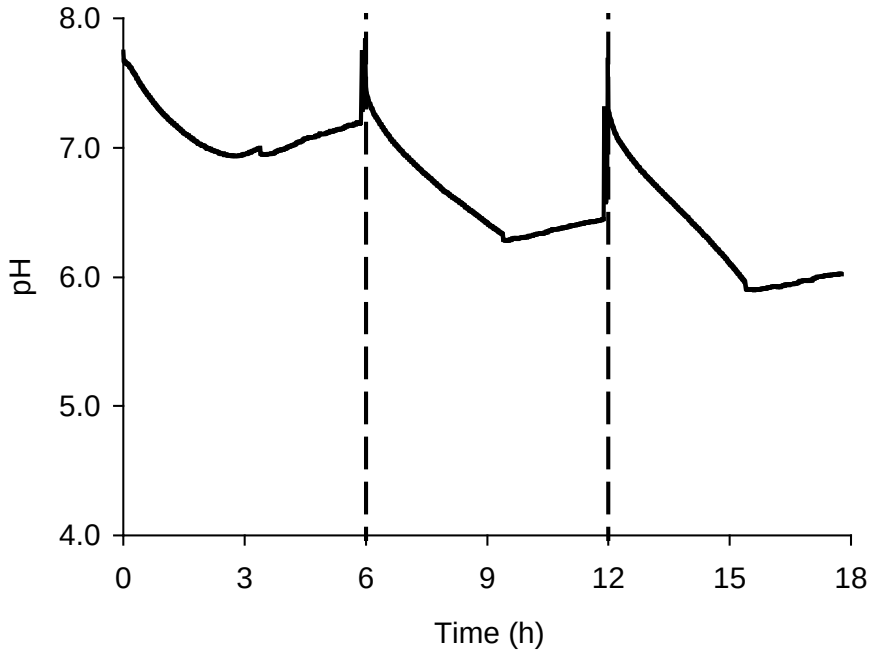

Figure 4.18 pH reduction in successive cycles of the nitrification trickling filter biofilm. Cycle length 360 minutes (150 minutes drain). Vertical lines indicate end of each cycle.

Addition of an extra 0.5 g L^{-1} sodium hydrogen carbonate to the nitrification feed supplied to the SBR containing approximately 2 g L^{-1} nitrifying biomass was sufficient to achieve a stable pH throughout ammonia oxidation (Figure 4.19). Addition of buffering to the synthetic wastewater would increase the cost of operation for the multistage system and may present a significant drawback of separating nitrification from denitrification biomasses.

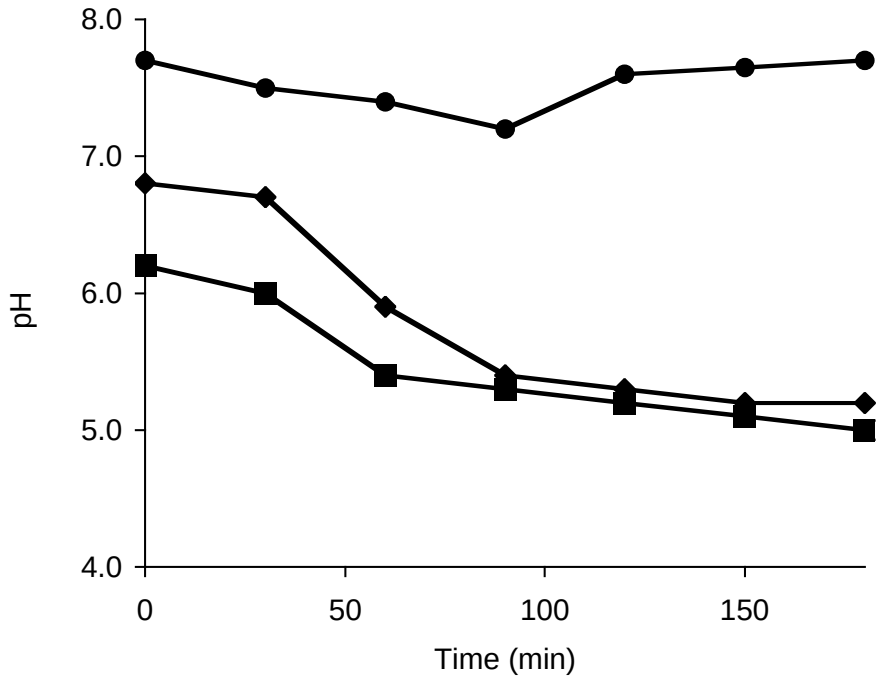

Figure 4.19 Effect of additional buffering to nitrification feed to maintain a suitable pH in the nitrification suspended growth SBR. Reactor k_La 45 ± 4.5 h^{-1}. No buffer addition (■), 0.25 g L^{-1} $NaHCO_3$ additional buffer (◆) and 0.50 g L^{-1} $NaHCO_3$ additional buffer (●). Cycle time 180 minutes.

4.3.4 Comparison of multistage system to published alternative reactors

The process on which the storage driven denitrification reactor was based, as described by Jones *et al* [40, 51], used a suspended culture SBR for storage of substrate by heterotrophic biomass. As a result, untreated ammonia was retained in the SBR and the

actual amount of nitrogen removed was limited (< 75%). In terms of nitrate alone, the multistage reactor in this study achieved the removal of nitrogen with a carbon supply of 3.3 kg COD/kg N-NO_3^- (based on Figure 4.16*A*). It is expected that this could be even lower as the nitrate removal did not seem to have reached a conclusion at the time of ceasing the experiment. If the retention of ammonia is taken into account, the carbon requirement observed increases to 4.2 kg COD/kg N-NO_3^- at an overall rate of 0.63 mM h^{-1}. Even with the ammonia retention, the multistage reactor performed competitively (Table 4.2) and appeared to have a lower requirement for carbon assimilation than in suspended growth reactors (Section 2.3.3). The specific data of overall nitrogen removal rate and C/N ratio of removal were not given in any of the studies found but in some cases could be calculated from published data (Section 4.3.5). As discussed before (Section 2.3.4), the rate data used for comparison requires sludge specific rate constants or similar to be truly transparent.

Table 4.2 Comparison of the multistage nitrogen removal reactor with other literature reactors/systems.

Reference	**This study**			**Jones [40]**	**Third [29]**	**Gibbs [41]**	**Bortone [48]**	**Zhang [99]**
	Figure 4.14	Figure 4.15	Figure 4.16					
Carbon requirement for nitrogen removal (kg COD/kg N)	13	8.0	4.2	32	17	11	8.1	2.7
Overall nitrogen removal rate (mM N h^{-1})	0.44	1.1	0.63	0.17	0.62	0.28	0.36	0.98

In terms of theoretical approach, the obvious alternative to the separation of the two different nitrogen removal biomasses is SND technology in which the needs of both microbial activities must be managed by careful oxygen control. Even when using PHB as electron donor for denitrification, the performance of the SND reactors is limited

compared to that developed here (Table 4.2). The studies of Third *et al* [29] and Gibbs *et al* [41] resulted in comparable overall rates of nitrogen removal but do not compete well in terms of carbon requirement for nitrogen removal (Table 4.2). The key reason is that when providing oxygen for nitrification, a significant amount of COD will be wasted through aerobic oxidation.

For those studies that also use separated nitrification and denitrification biomass to achieve complete nitrogen removal, a range of results was observed (Table 4.2). In those with substantial demand for COD to achieve both nitrogen and phosphorus removal, there was an increase C/N ratio of removal [48].

4.3.5 Proposal of criteria for the comparative assessment of wastewater treatment strategies wastewater treatment processes

It is difficult to directly compare the nitrogen removal performance of different systems using a single criterion such as percent or fraction of nitrogen removed. For critical appraisal of treatment approaches, it is important that a number of robust and transparent performance measures are evaluated simultaneously. Previous researchers in the field of SND development have suggested the use of a multiplication of the rate of SND achieved and the fraction of SND achieved [39]. The work in question investigated the effect of oxygen set point on the SND performance using a consistent C/N ratio feed. As such, the measure was adequate for that circumstance but does not address non nitrogen removing periods such as COD sequestering, nor the higher level of carbon limitation observed in this thesis.

Criteria that address performance in a relatively objective way are the C/N treatment ratio, that is, the relative amount of carbon and nitrogen actually removed by the process, and the overall rate at which the nitrogen removal was achieved. It is important that the overall rate of nitrogen removal as an average of the whole process is considered rather than the individual rates of nitrification, denitrification or other phase. Any nitrogen removal idle periods, such as in the storage driven denitrification reactor where the acetate uptake phase does not substantially contribute to nitrogen removal, still contribute to the cost and performance of the system and therefore need to be considered. In this treatment system, despite high rates of denitrification and nitrification these do not necessarily reflect the rate of nitrogen removal overall.

The C/N ratio of removal of the system encapsulates a cost component indirectly. The delivery of oxygen to a mixed sludge culture with access to a high COD and N feed will cause the wastage of oxygen and organic carbon. Subsequently, nitrogen removal may be limited and more COD often needs to be added to complete nitrogen removal. Where native COD is wasted by aerobic metabolism in this way, less nitrogen will be treated than where aerobic metabolism is not allowed to occur. The C/N ratio of removal is a direct reflection of the efficiency of carbon use. The C/N ratio of removal is also a more reliable and transparent measure of nitrogen removal than fraction (or percent) of nitrogen removed as the amount of nitrogen removal that can be achieved depends directly on the amount of carbon available. As a result, fraction of nitrogen removal can be misleading if the carbon in the feed supplied was generous.

Even though C/N ratio of removal and overall nitrogen removal rate are both good measures of performance they do not adequately indicate optimal performance individually. Thus, an approach based on a matrix comparing different systems on both

criteria is preferable (Figure 4.20). The best performing, or optimal, systems would be those with a low C/N ratio of removal that perform at relatively high rates of nitrogen removal while those with high C/N ratio of removal and low rates are performing inefficiently. The relative value of the C/N ratio of removal could be based on the hypothesised minimum of COD requirement for nitrogen removal (Appendix A).

		C/N ratio (kg COD/kg N)	
		< 4.70 kg COD/kg N	**< 4.70 kg COD/kg N**
Overall N removal rate (mmole N/L/h)	**< 0.5 mmole N/L/h**	adequate	inefficient
	> 0.5 mmole N/L/h	optimal	adequate

Figure 4.20 Proposed matrix for assessment of performance of wastewater treatment systems considering the C/N treatment ratio and overall rate of nitrogen removal.

In addition, this approach is more instructive than artificially creating a single evaluation tool by simple multiplication of C/N ratio and rate of nitrogen removal. The matrix would allow operators to transparently assess a process and identify where improvements could be made either by better carbon conservation or improved rate. The usefulness of a direct multiplication would be better if N/C ratio of removal was used instead of C/N ratio of removal as the most optimal performance would have a high value for both rate and N/C ratio of removal. In this case a direct multiplicative measure would be a good single evaluative tool for the overall performance but would still not reveal information about possible targeted improvement of the system in question.

The specific rate of nitrogen removal would be a further useful generalisation that is highly recommended. Due to the difficulty in determining a representative sample of

biomass in the separated biofilm reactors, this was not feasibly achieved in this study. It is recognised to be an avenue for further improvement in the quality of communication in wastewater treatment publications not undertaken in this study.

Percent of nitrogen removed could form an additional dimension in the testing matrix but for the reasons discussed above, this would be insufficient alone to critically evaluate the treatment performance and is, essentially included in the C/N ratio whenever feed composition is known. For the storage driven denitrification biofilm system developed here, the C/N treatment ratio was very competitive while the overall nitrogen removal rate was favourable (Table 4.2).

4.4 Conclusions

The combination of the storage driven denitrification biofilm with a nitrification reactor has allowed the development of a multistage wastewater treatment system in which competitive carbon requirement for nitrogen removal and high overall nitrogen removal rates were achieved. However, the nitrogen removal capacity of the multistage system was limited by the physical retention of ammonia in the storage driven denitrification reactor. In addition, the decrease in pH during nitrification reduces the activity of nitrifiers unless additional buffer was added. These issues need to be addressed before the multistage system could be developed into a full scale treatment system.

Chapter 5. Parallel nitrification-denitrification: An alternative to multistage treatment

Abstract

Nitrogen removal from wastewater involves aerobic nitrification and denitrification. To minimise the aerobic oxidation of COD, which is required as electron donor for denitrification, a three-stage two-biomass system had been described involving separated biofilms for nitrification and denitrification and strictly sequential operation. The anoxic biofilm stored COD as PHB, allowing a separate aerobic biofilm to exclusively oxidise ammonia to nitrate, which is in the third sequence fed back to the storage reactor for denitrification (storage driven denitrification). However, pH drops from 7 to 5.5 in the nitrification culture, which inhibited nitrification. In addition, the retention of untreated ammonia from the storage driven denitrification reactor limited nitrogen removal to less than 80%. A new separated sludge based column bioreactor system with simultaneous liquor cycling through the above nitrification and denitrification columns was developed. Rather than treating the artificial wastewater sequentially it continuously recycled it between the nitrification and denitrification biofilms, avoiding completely the acidification observed previously and improving the nitrogen removal to levels not reported in literature. Even at very low feed C/N ratios between of 2.0 and 3.4 kg COD/kg N-NH_3, >99% nitrogen could be removed at overall rates of 0.4 to 0.9 mM N h^{-1}, largely due to the avoidance of aerobic COD oxidation and the short cut through nitrite between nitrification and denitrification. Even though particularly low feed C/N ratios were used, the storage driven denitrification biofilm could still store reducing power enabling repeated nitrate feeds to be reduced. A series of tests with real wastewater showed a carbon requirement of 7.6 kg COD/kg N-NH_3 treated.

5.1 Factors limiting multistage nutrient removal treatment systems

The advantages of separating nitrifying and denitrifying biomasses for nutrient removal in wastewater treatment are numerous. Principal among these is the ability to customise the delivery of electron acceptors and donators specifically as required. This separation allowed for optimised growth and performance of nitrifiers and denitrifiers with minimal waste of both oxygen and COD supply, since the aerobic oxidation of COD was avoided (Section 2.3.3). Using this dual biomass approach by an entirely sequential operation of anoxic storage (reactor 1), aerobic nitrification (reactor 2) and anoxic denitrification (reactor 1), good nitrogen removal rates of up to 1.1 mmole NH_3/L/h were observed in the multistage reactor with a relatively low supply of electron donor (COD). The COD requirement for N removal was about 3.9 kg COD/ kg N-NH_3. The system was limited, however, in performance and cost by several intrinsic technical problems. As the multistage system required sequential separated nitrification and denitrification, it also required a holding tank to prevent mixing of the partially treated wastewaters between stages.

As expected, the nitrification reactor, regardless of design (trickling filter or SBR) produced a detrimental shift in pH. Depending on specific activity level, a drop of up to 0.9 pH units per hour has been observed, resulting in pH drops from 7.0 to 5.5 (Section 4.3.3). As free ammonia (NH_3) rather than ammonium (NH_4^+) is the principal substrate of nitrification, it is strongly inhibited by acidic pH conditions and so the drop in pH reduced the specific activity of the bacteria, an effect that is cumulative over successive cycles (Section 4.3.3). The continual need for pH control is known to be a significant

cost factor in wastewater treatment and should be avoided, in particular when considering that the acid producing nitrification sequence is followed by an acid consuming denitrification sequence. A direct comparison of the buffer capacity of a real wastewater and the synthetic analogue used in this study (Section 4.3.3) revealed that a real wastewater may be less able to counteract such a pH drop than the synthetic analogue.

All dual biomass systems with sequential operation not only face the pH problem described above but are also limited by the demonstrated physical retention of ammonia in the storage driven denitrification reactor during acetate uptake (Appendix E) as it caused the release of untreated ammonia in the final stage effluent from the denitrification phase. This was a major physical limitation to the amount of nitrogen such a system was able to treat, restricting it to a maximum nitrogen removal efficiency that corresponded to how much liquid is retained in the biomass sludge after separation of bulk liquid from biomass. When using traditional sludge settling with 50% settled sludge volume, only 50% of nitrogen can be removed [37]. By the sequential system using a storage driven denitrification biofilm the separation was more efficient allowing about 80% nitrogen removal ([84] and Chapter 4). To reduce ammonia retention, backwashing would need to be introduced between the acetate uptake and denitrification phases to release ammonia for treatment by the nitrification reactor. Additional liquid flow may require additional holding tanks and increase the overall cost of operation.

The aim of this paper was to find a solution that minimised or eliminated the three issues described above (pH drift, holding tank requirement and ammonia washout) based on the previously described multistage sequential wastewater system with

separated storage driven denitrification and nitrification biomasses ([84] and Chapter 4). The new approach described here still uses a dual biomass system with an aerobic nitrification biofilm in one reactor and a storage driven denitrification biofilm in the second reactor, minimising contact of COD and oxygen. Further, the system still uses an initial sequence of storing COD as poly-β-hydroxybutyrate (PHB), but it then circulates the COD free solution continually between the two reactors, allowing aerobic ammonia oxidation to nitrate in the second reactor at the same time as anoxic storage driven nitrate reduction to N_2 in the first reactor. Because of the simultaneous rather than sequential operation of nitrification and denitrification the system is termed Parallel Nitrification and Denitrification (PND).

5.2 Materials and Methods

5.2.1 Laboratory reactor design and operation

The PND reactor design made use of the two separate biofilm reactors (storage driven denitrification and nitrification) described earlier as the multistage system ([84] and Chapter 4) in a way that eliminated the holding tanks between phases. Initially, during the storage phase, the artificial wastewater was added (using about 3% of the treatment time) and recycled (Figure 5.1) through the storage driven denitrification biofilm to accomplish anoxic COD uptake and storage as PHB. This was accomplished by an upflow and using a small sump (recycle vessel) to collect the outflow from the top of the reactor. After the COD of the original synthetic wastewater was successfully stored in the storage driven denitrification reactor, as previously described, the resulting COD

free, ammonia containing wastewater was not only recycled through the one reactor but the outflow from the recycle vessel was split such that about 50% passed through the denitrification biofilm reactor while the other 50% was passed through the nitrification biofilm reactor. The nitrification reactor was operated in a downflow mode such that the biomass was exposed to oxygen containing atmosphere at all times (trickling filter reactor, TF). The recycle vessel had ports for DO, pH and Eh probes and also inlets and outlets for the two reactors (Figure 5.1). In addition, the nitrification trickling filter had a port for head space DO measurement and needle ports for controlled air delivery and pressure equalisation. For parallel operation, each reactor had an independent pump that could be separately controlled allowing manipulation of the flow rate to each of the reactors. The system was operated under parallel nitrification and storage driven denitrification until the end of the pre-determined cycle and all liquid was drained rapidly followed by a new fill cycle to the storage driven denitrification reactor.

Very high dissolved oxygen concentrations (> 4 mg L^{-1}) were observed in the recycle vessel during initial experiments. To prevent oxygen transfer to the denitrification reactor, the design was modified. In the modified design, the air flow was controlled to the reactor by a feedback control that left the DO concentration in the recycle vessel less than 0.1 mg L^{-1}.

Unless otherwise specified, the feed supplied to the reactor was the same as that used for the multistage system (Section 4.2.1). For particular experiments, an actual municipal wastewater stream was sought. The source was the effluent from the primary treatment of the Woodman Point Wastewater Treatment Plant. The sample was analysed thoroughly prior to use showing 500 mg L^{-1} COD, 4 mM ammonia and 1 mM acetate (Table 2.1).

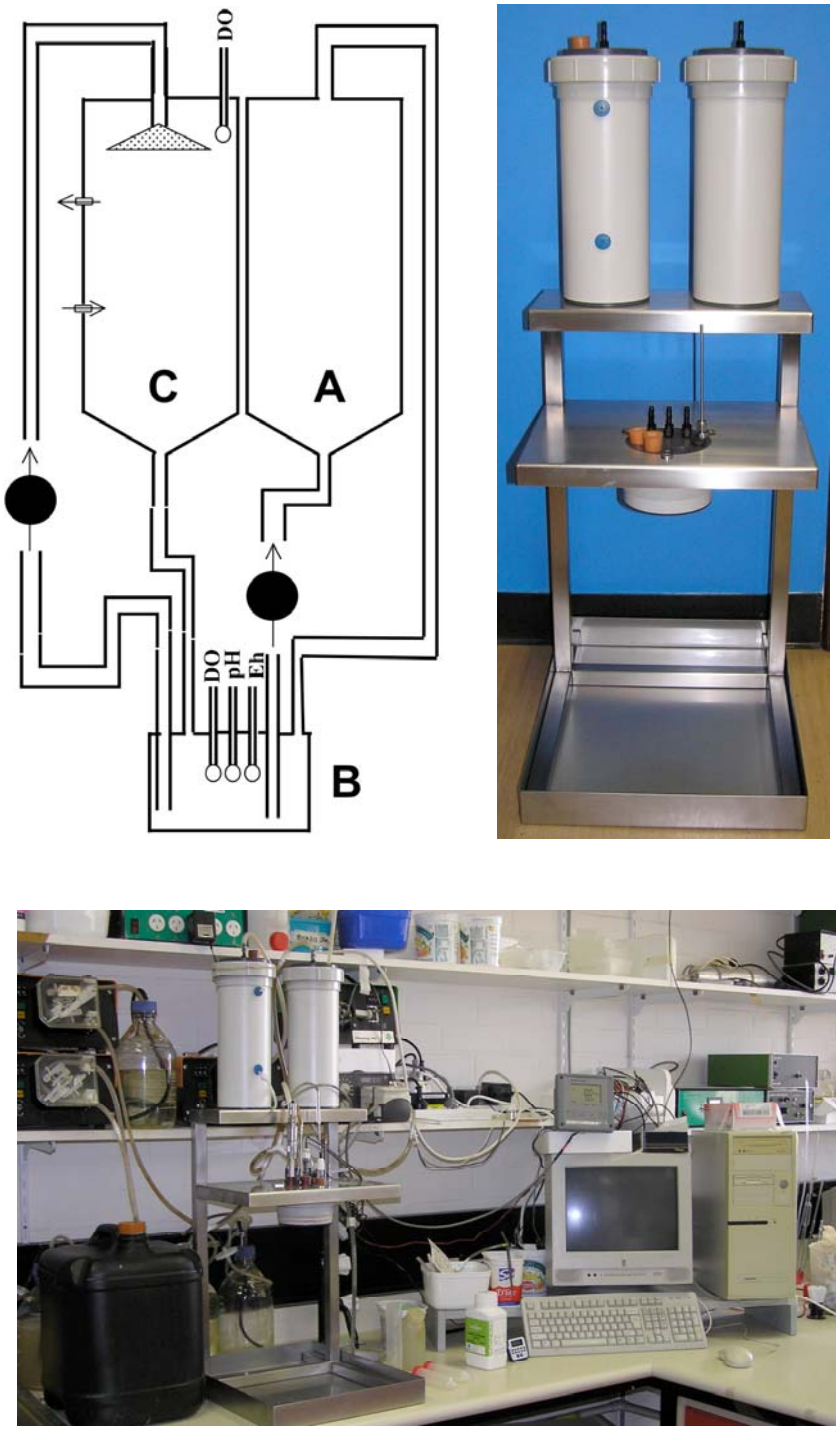

Figure 5.1 PND reactor where, after the initial batch acetate uptake phase where liquid only recirculates through the denitrification reactor, the nitrogen rich liquor is passed simultaneously from the recycle vessel to each of the denitrification and the nitrification reactors and back into the recycle vessel. This experimental setup was used for all experimental work in Chapters 5, 6 and, unless otherwise specified, Chapter 7. Schematic shows the storage driven denitrification biofilm reactor (A), the recycle vessel (B) and nitrification trickling filter reactor (C). The recycle pumps are indicated by solid circles with arrows for direction of flow. Photos show the unconnected and operating PND system.

5.2.2 Analysis

All nitrogen and phosphorus compounds were measured spectrophotometrically (Section 2.2.4). The acetate and PHB content of lyophilised biomass was measured by gas chromatography (Section 2.2.4) and glycogen content of lyophilised biomass was measured by either spectrophotometry or with a YSI glucose analyser (Section 2.2.4).

5.2.3 Calculations

Measurement of k_La, calculation of OUR and hence oxygen consumed, C/N ratios of removal and overall rates of nitrogen and ammonia removal were performed as described previously (Sections 2.2.6 and 4.2.4).

Rates of nitrification and denitrification

The rate of ammonia oxidation during the PND phase was calculated from the amount of ammonia removed during the time of PND phase operation. Where nitrate or nitrite accumulated, the denitrification rate was estimated from the difference between the ammonia oxidation rate and the nitrate and nitrite accumulation rate.

5.3 Results and Discussion

5.3.1 Does PND operation eliminate the intrinsic technical problems of the multistage nutrient removal process?

The multistage nutrient removal process described previously ([84] and Chapter 4) showed good biological nutrient removal potential, but was limited by the need for holding tanks, the detrimental drop in pH during nitrification and the release of untreated ammonia from physical retention during acetate uptake. The simultaneous flow of the COD depleted, high ammonia feed after acetate uptake between the nitrification and denitrification reactors in the PND system was tested as a possible solution to all three problems. The simultaneous flow of liquor between the two reactors eliminated the need for a holding tank between treatment stages as it was no longer necessary for the different partially treated wastewaters to be separated. In terms of implementation of this system to an industrial scale, this should lower the overall footprint of each treatment unit.

The usual pattern of pH change under multistage conditions resulted in the lowering of pH during nitrification, which substantially inhibited the activity level of the nitrifiers (Section 4.3.3). During PND operation, the pH of the reactor liquor decreased (Figures 5.2 and 5.3), however the magnitude of the decrease was substantially reduced and an inhibitory effect on nitrification was not observed. The PND phase of the observed cycles had an average decrease in pH of 0.09 units h^{-1} while the nitrification reactor had an average decrease of 0.47 units h^{-1}.

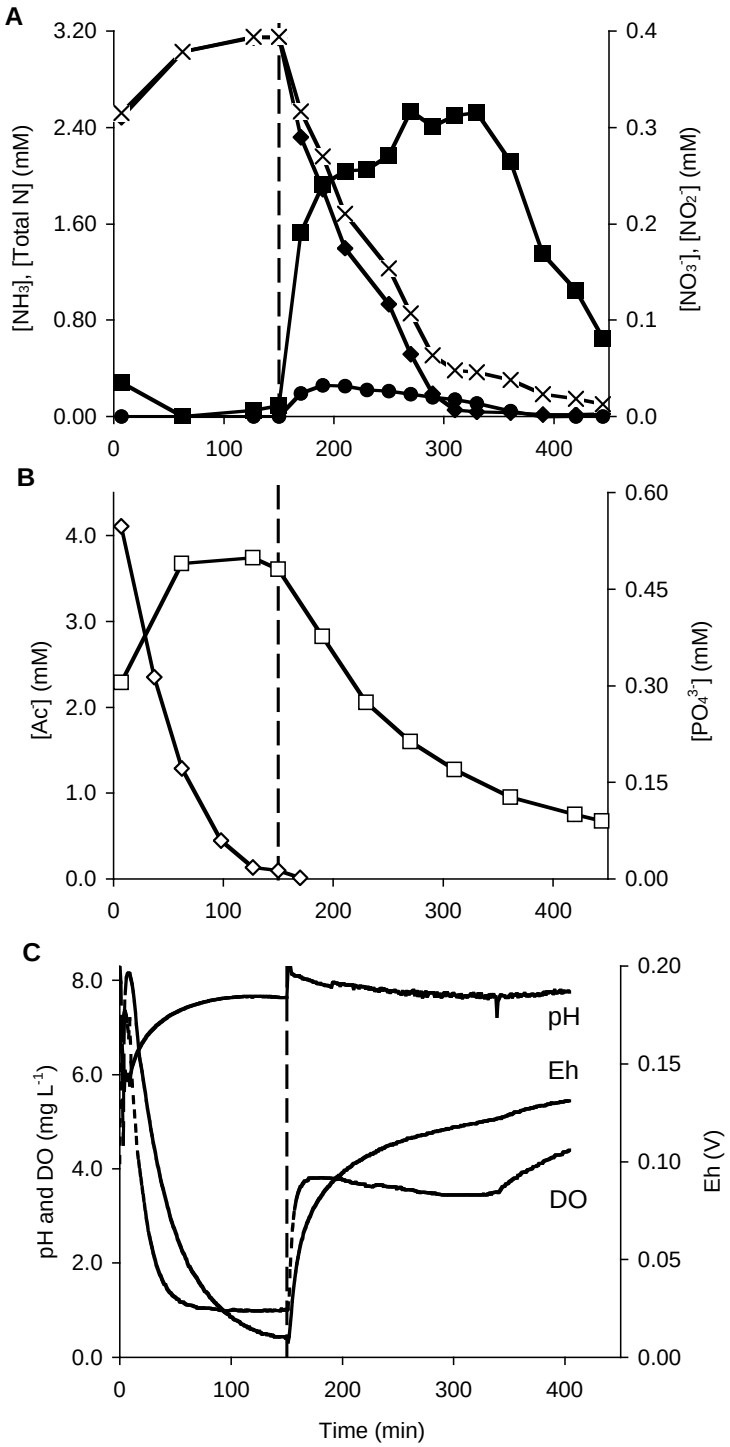

Figure 5.2 Effect of cycling wastewater simultaneously through nitrification and denitrification biofilm reactors after COD removal. N compound (A), acetate and phosphate (B), DO, pH and Eh (C) behaviour observed during the first trial of four sequential trials under PND operating conditions with synthetic wastewater (Feed Ammonia 2.5 mM and Acetate 4.1 mM; 7.6 kg COD/kg N-NH_3): Acetate (◇), phosphate (□), ammonia (◆), nitrate (■), nitrite (●) and total nitrogen (×). Vertical line indicates phase change from acetate uptake phase (130 minutes) to nitrogen removal phase (350 minutes)

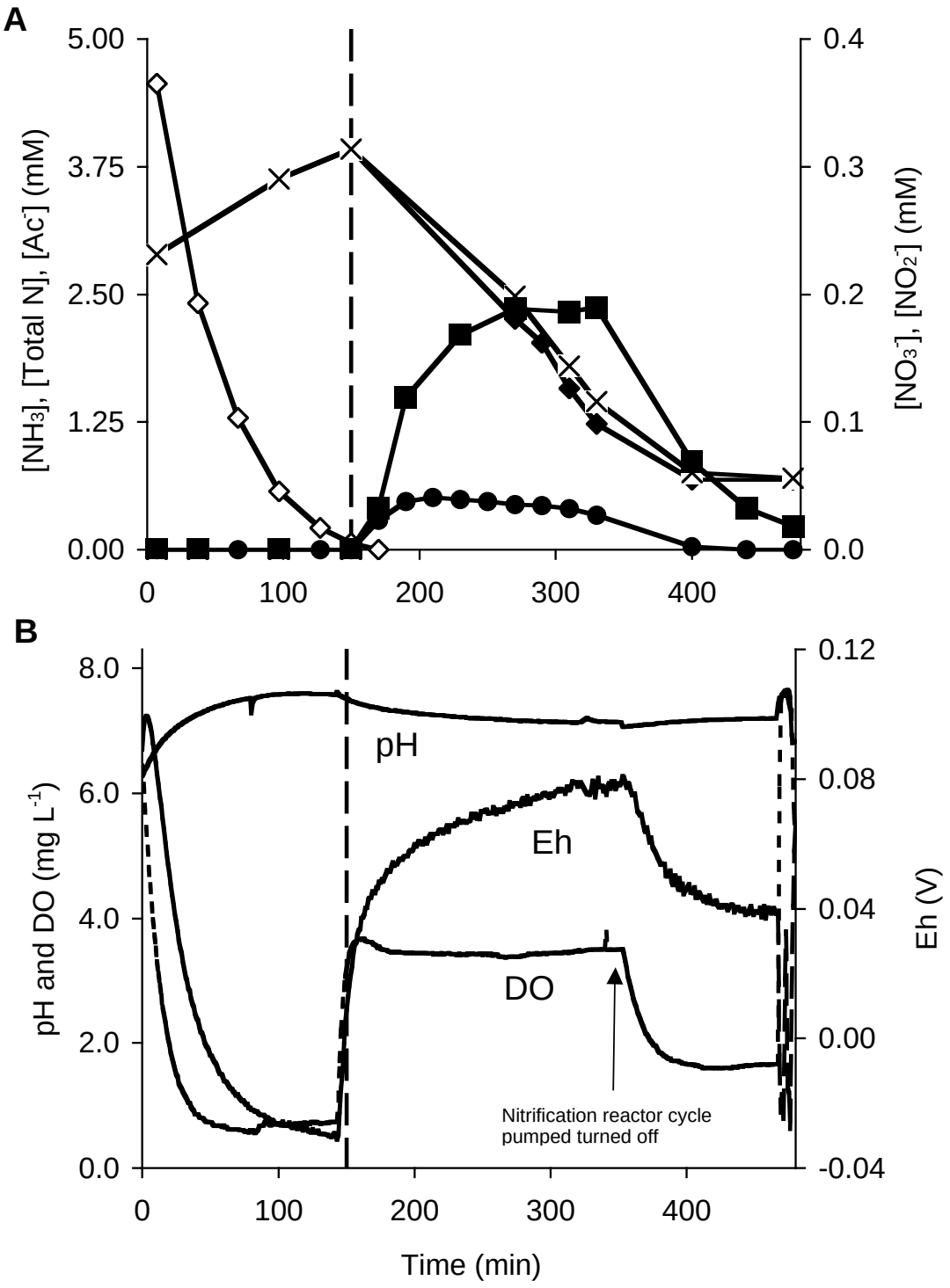

Figure 5.3 Reproducible effect of cycling wastewater simultaneously through nitrification and denitrification biofilm reactors after COD removal. N compound (A), acetate and phosphate (B), DO, pH and Eh (C) behaviour observed during the first trial of four sequential trials under PND operating conditions with synthetic wastewater (Feed Ammonia 2.9 mM and Acetate 4.6 mM; 7.2 kg COD/kg N-NH_3): Acetate (◇), phosphate (□), ammonia (◆), nitrate (■), nitrite (●) and total nitrogen (×). Vertical line indicates phase change from acetate uptake phase (130 minutes) to nitrogen removal phase (350 minutes).Arrow indicates time at which nitrification recycle pump turned off.

When nitrification occurs simultaneously with denitrification, the denitrifiers consume a portion of the protons produced *via* nitrification [101] as shown in the following equations, omitting assimilation. A fraction (0.375 of 2, or 19%) of the protons produced during nitrification should be consumed by the denitrifying organisms and thus partially counteract the pH drop.

$$NH_4^+ + 2\ O_2 \longrightarrow H_2O + NO_3^- + 2\ H^+ \qquad \text{Equation 5.1}$$

$$NO_3^- + 0.625\ CH_3COO^- + 0.375\ H^+ \longrightarrow 1.25\ HCO_3^- + 0.5\ H_2O + 0.5\ N_2 \qquad \text{Equation 5.2}$$

The effect of combining nitrification with denitrification for reduced pH depression would have an impact on the pH of the system according to the stoichiometry above, shown adjusted for the dominant species at the usual operating pH of the reactor (between pH 7-8). Given the only marginal drop in pH by operating under PND conditions (-0.09 pH units h^{-1}) compared with the multistage system (-0.47 pH units h^{-1}), it was clear that the concurrent operation of nitrification and denitrification stabilised the pH. External operator control of pH was not necessary.

In contrast to sequential nitrification and storage driven denitrification, the parallel nitrification and storage driven denitrification showed no residual ammonia present at the end of the PND phase (Figure 5.2).

It is unclear which chemical species is the dominant oxidised nitrogen that transits between the nitrification and denitrification reactors as both nitrite and nitrate are detected in the recycle (Figures 5.2 and 5.3) and both could be effectively reduced by the denitrification biofilm. The fact that concentrations of nitrate were higher than

nitrite suggested that nitrate was the major transition species between the two reactors during the PND phase.

Both nitrate and nitrite were observed at concentrations considerably less than the total nitrogen in the system at the start of the PND phase. Nitrate accumulated to a maximum of 10% of the total nitrogen present at the beginning of the cycle while nitrite only accumulated to 1% of the total nitrogen present at the beginning of the cycle (Figures 5.2 and 5.3). The stability of the pH coupled with the considerably less than stoichiometric transfer of ammonia to either nitrate or nitrite is evidence of nitrification and denitrification acting in concurrence due to the direct cycling of fluid between the reactors.

5.3.2 Nutrient removal capacity of the PND system

The data above suggest that the PND system is superior to the multistage system in terms of managing the technical issues associated with separated nitrification and denitrification biomasses. To challenge the system, considerably lower feed C/N ratios were tested. Theoretically, if all added COD is used for nitrate reduction to N_2 and nitrogen assimilation is ignored the lowest treatable C/N ratio is 2.9 kg COD/kg N (Appendix A). When operating the PND system with a feed C/N ratio of about 3.9 kg COD/kg N-NH_3, nitrification and denitrification occurred in parallel without the buildup of significant nitrate or nitrite (Figure 5.4). When doubling the concentrations of both ammonia and COD (acetate) and providing a correspondingly longer treatment time denitrification was still able to run in parallel with nitrification (Figure 5.5). By further reducing the feed C/N ratio to 2.4 kg COD/kg N there were was a small

tendency of nitrate build-up, but not to significant levels (Figure 5.6). After increasing the COD and ammonia concentration 3-fold while still leaving the feed C/N ratio at about 2.5 the PND system still resulted in complete N removal to about 96% (Figure 5.7 and Table 5.1). Hence the carbon requirement for nitrogen removal varied between 2.4 and 4.0 kg COD/kg N, which could be the result of a shortcut nitrite shunt or luxury storage.

In general, it appeared that carbon requirement could be improved by simply lowering the C/N ratio of the feed (Table 5.1). The tests in which the feed C/N ratio was lower also achieved a lower carbon requirement. While this may be counterintuitive based on previous data where a lower C/N ratio increases the proportion of carbon used for assimilation (Appendix E), it actually implies that too much carbon was routinely being fed to the system.

The competitive carbon requirement observed in at least one case (2.6 kg COD/kg N in Figure 5.6 and Table 5.1) surpassed that of the stoichiometric minimum for nitrate reduction (2.9 kg COD/kg N). There are two possible explanations for this result. The link between nitrification and denitrification is *via* the nitrite shunt (Figure 5.8), a concept well described for simultaneous nitrification and denitrification [19, 41, 98]. The flow of liquor between the two reactors may have provided an opportunity to exploit this short circuit which saves up to 40% COD (Figure 5.8). As a result, a substantial amount of ammonia fed to the PND reactor may have been oxidised to nitrite and then directly reduced to nitrogen in the denitrification reactor.

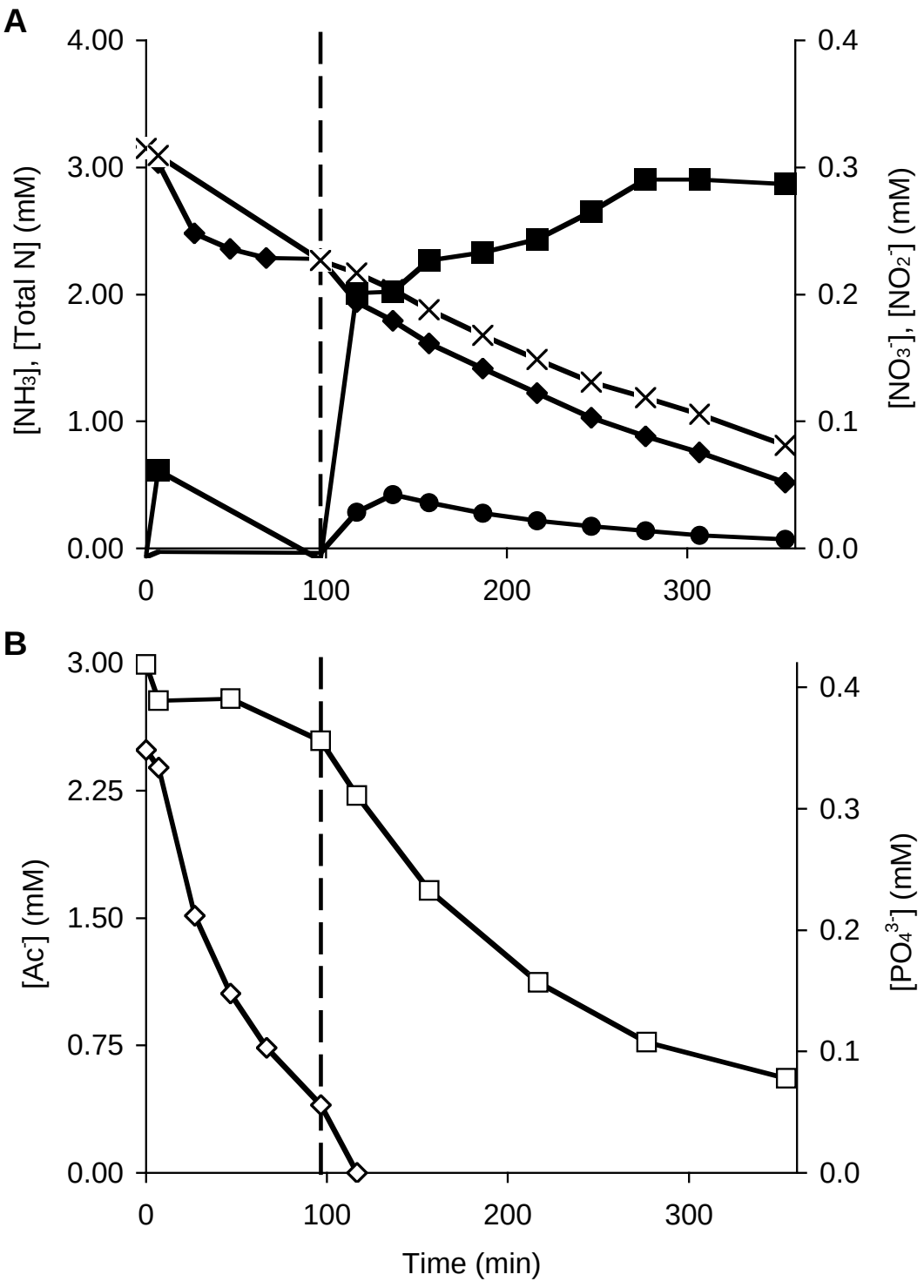

Figure 5.4 Effect of lowering supply of COD on nitrogen removal (first of four tests). N compound (A), acetate and phosphate (B) results for PND operation fed synthetic wastewater (Feed ammonia 3.16 mM and acetate 2.49 mM; 3.89 kg COD/kg N-NH_3): Acetate (◇), phosphate (□), ammonia (◆), nitrate (■), nitrite (●) and total nitrogen (×). Vertical line indicates change of phase from acetate uptake (95 minutes) to nitrogen removal (265 minutes).

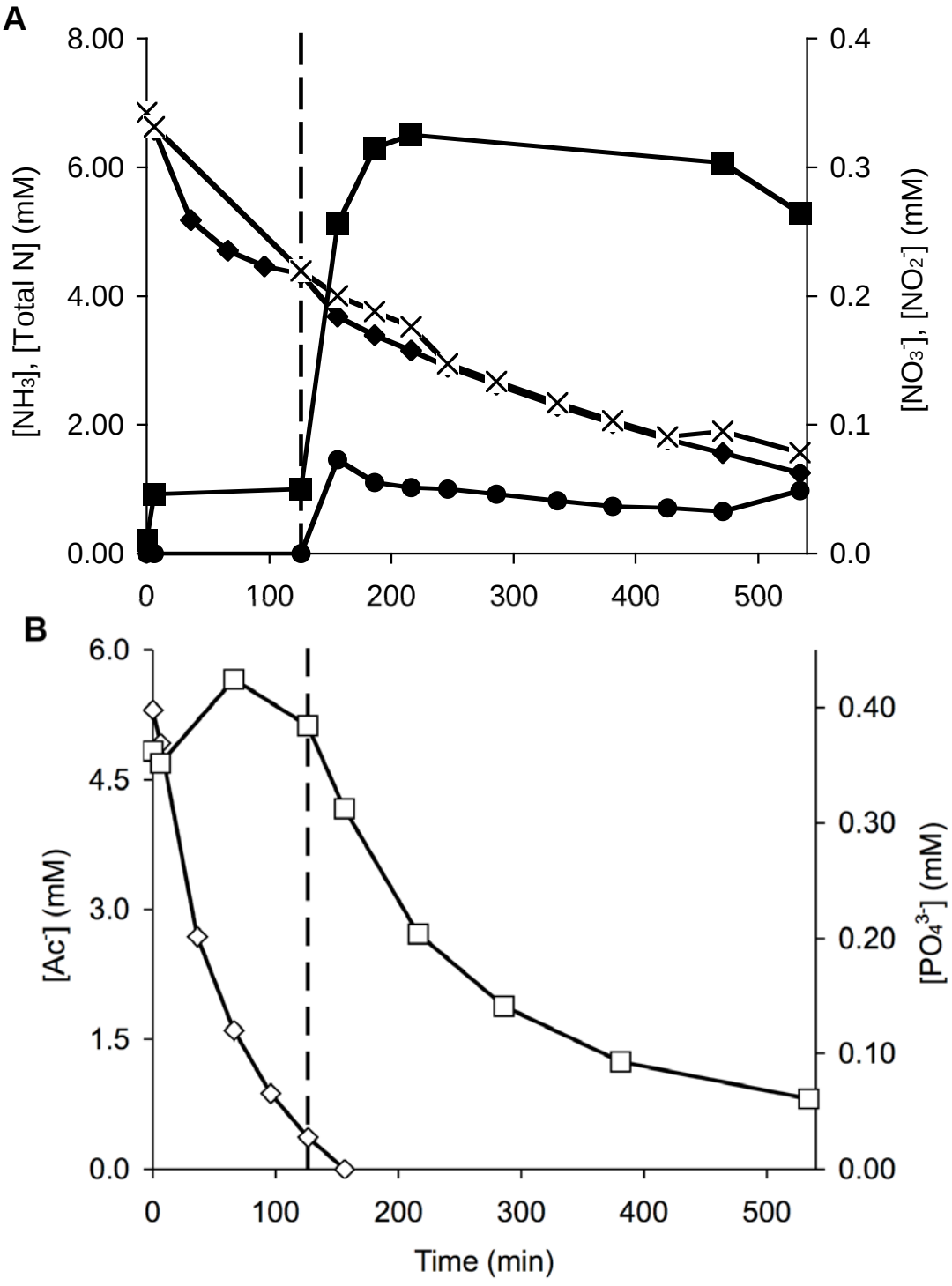

Figure 5.5 Effect of lowering supply of COD on nitrogen removal (second of four tests). N compound (A), acetate and phosphate (B) results for PND operation fed synthetic wastewater (Feed ammonia 6.8 mM and acetate 5.3 mM; 3.8 kg COD/kg N-NH_3): Acetate (◇), phosphate (□), ammonia (◆), nitrate (■), nitrite (●) and total nitrogen (×). Vertical line indicates change of phase from acetate uptake (130 minutes) to nitrogen removal (410 minutes).

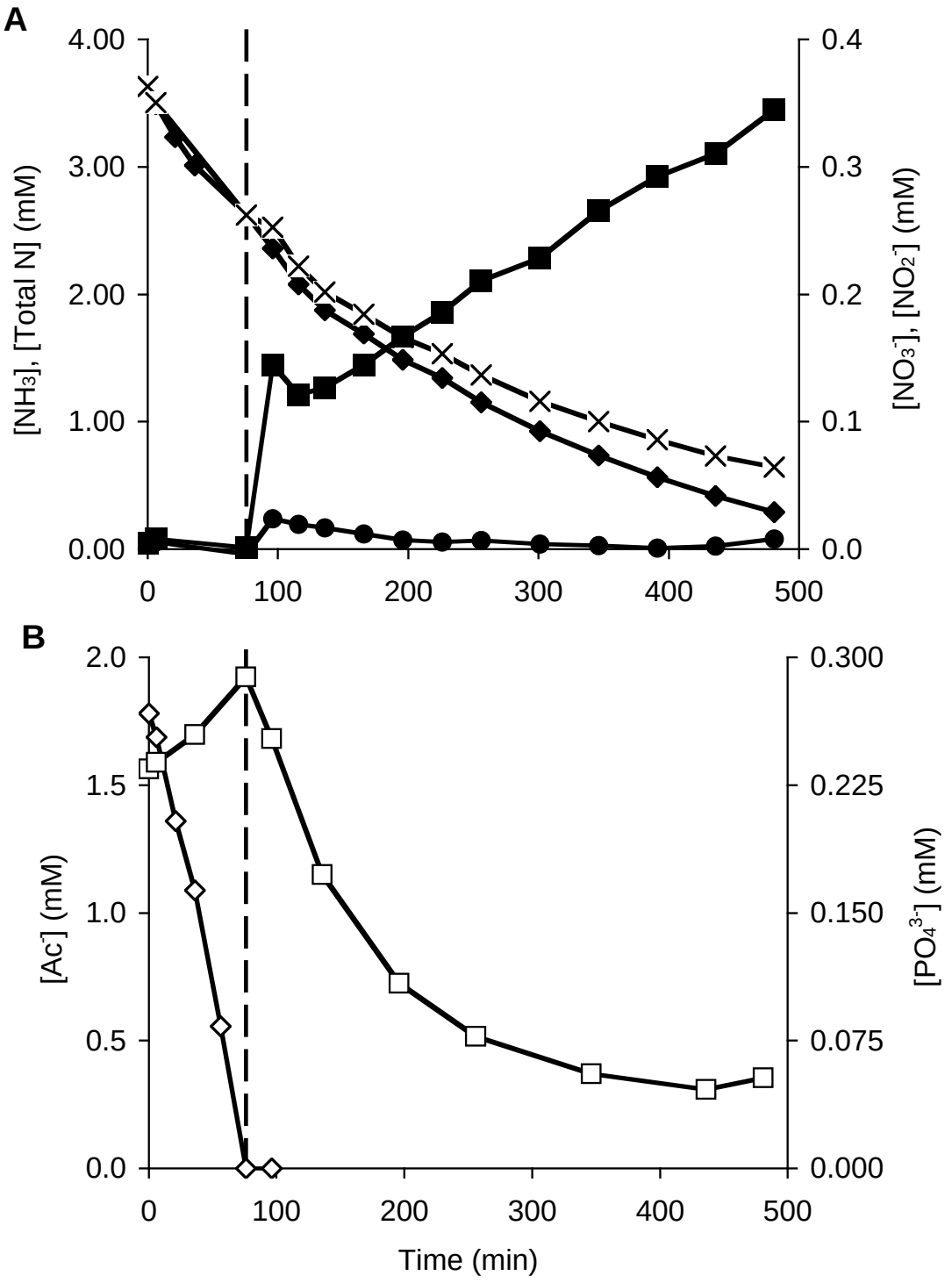

Figure 5.6 Effect of lowering supply of COD on nitrogen removal (third of four tests). N compound (A), acetate and phosphate (B) results for PND operation fed synthetic wastewater (Feed ammonia 3.6 mM and acetate 1.8 mM; 2.4 kg COD/kg N-NH_3): Acetate (◇), phosphate (□), ammonia (◆), nitrate (■), nitrite (●) and total nitrogen (×). Vertical line indicates change of phase from acetate uptake (70 minutes) to nitrogen removal (410 minutes).

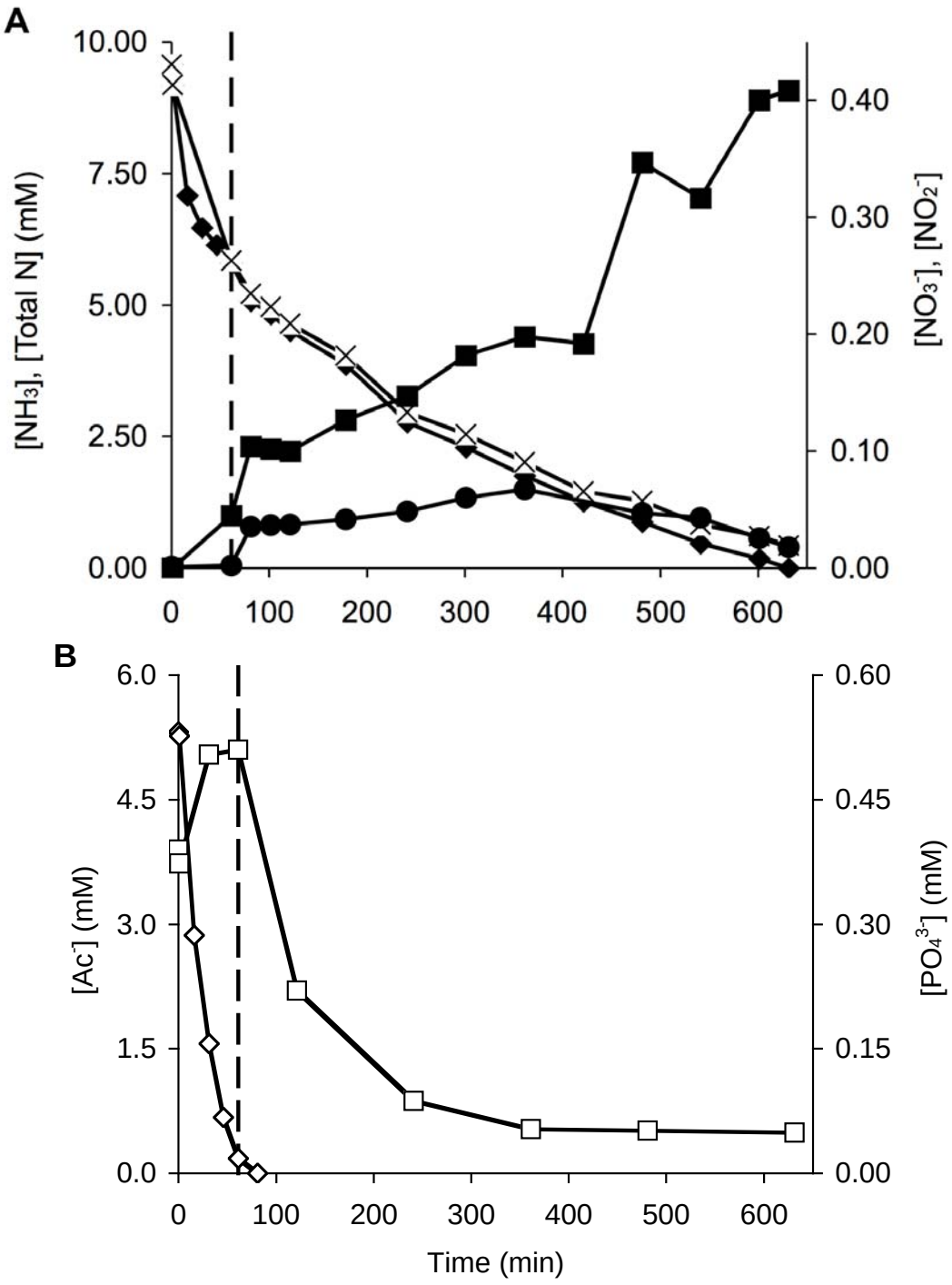

Figure 5.7 Effect of lowering supply of COD on nitrogen removal (fourth of four tests). N compound (A), acetate and phosphate (B) results for PND operation fed synthetic wastewater (Feed ammonia 9.6 mM and acetate 5.3 mM; 2.5 kg COD/kg N-NH_3): Acetate (◇), phosphate (□), ammonia (◆), nitrate (■), nitrite (●) and total nitrogen (×). Vertical line indicates change of phase from acetate uptake (60 minutes) to nitrogen removal (570 minutes).

Table 5.1 Summary of key results from testing of possible C/N treatment ratio of the PND system.

Feed C/N (kg COD/kg N)	Carbon requirement for nitrogen removal (kg COD/kg N)	%N removal overall	%N removal during Ac^- removal	Initial $[NH_3]$ (mM)	Overall [16]rate of N removal (mM N h^{-1})	Initial $[Ac^-]$ (mM)	Initial rate of $[Ac^-]$ removal (mM N h^{-1})
3.9	5.6	84	28	3.2	0.40	2.5	2.18
3.8	4.6	77	36	6.8	0.59	5.3	4.35
2.4	2.9	82	28	3.6	0.37	1. 8	1.20
2.5	2.6	96	39	9.6	0.87	5.3	9.06

Alternatively, as supported by other data (Section 5.3.5), the routine excess supply of carbon to the PND reactor may have resulted in luxury storage of acetate for use in cycles in which the supply of carbon was insufficient. It is unlikely that this was a major factor in these experiments, however as each resulted in the accumulation of nitrate (Figures 5.6 and 5.7). If substantial intracellular carbon was available from previous cycles, any nitrate produced should have been reduced to nitrogen gas. However, as previous cycles were all run with relatively low C/N ratios, excessive storage of reducing power could be excluded.

[16] Overall rate is taken as the rate of nitrogen removal over the entire length of the experiment including the acetate uptake phase.

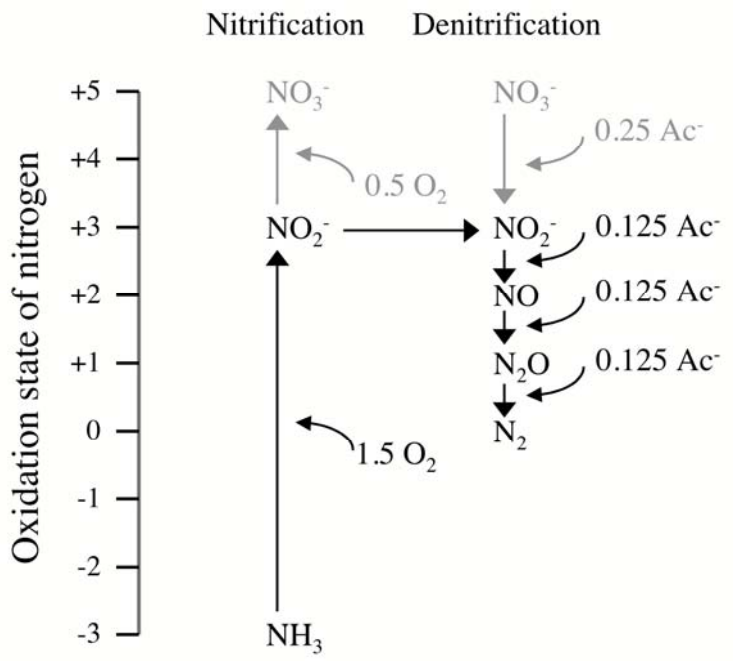

Figure 5.8 Theoretical short circuit between nitrification and denitrification providing savings of 40% COD (0.25 mol acetate/0.625 mol acetate) and 25% oxygen (0.5 mol oxygen/2 mol oxygen) as a result of nitrification-denitrification *via* nitrite.

Evaluation of overall nitrogen removal rate of PND reactor

The low levels of nitrate and nitrite show that the nitrification rate was rate limiting with denitrification being nitrate/nitrite limited. Hence, the rate of denitrification was closely matched to that of nitrate (or nitrite) production from oxidation of ammonia, (Figures 5.2 – 5.7). The overall rate of nitrification removal by the PND system was similar to that observed in the multistage system [84], generally in the range 0.4 – 0.9 mM h^{-1} (Table 5.1).

Together with the removal of nitrogen, the PND system showed a reproducible phosphate uptake phase during the PND phase. The fact that a similar first order type phosphate uptake kinetics was observed in the storage driven denitrification reactor of the sequential multistage system described (Chapter 4 and [84]) suggests that phosphate uptake is accomplished by the denitrifying bacteria. From the amount of phosphate

taken up it can be concluded that it was not assimilated but stored as described for PAOs [102-104]. This is supported by the observation that phosphate was released, presumably from polyphosphate hydrolysis as an energy source, during the acetate uptake period. The PND system eliminated the intrinsic technical issues associated with spatially separated nitrifiers and denitrifiers, such as pH depression, while maintaining or improving the key performance criteria for efficient wastewater treatment.

5.3.3 Comparison of PND system to similar approaches

The data above support the competitiveness of the PND approach to the multistage system. The multistage system has been compared substantially to alternative reactor designs and achieved comparatively good nutrient removal ([84] and Chapter 4). Performance data from literature studies of separated biomass reactors is limited, and reported treatment efficiencies generally do not include influent C/N ratios or carbon requirement for nitrogen removal. Similar systems to PND in approach exist in the literature, however none specifically include conservation of influent COD by intracellular storage [105] (Table 5.2). Instead, pre-denitrification is used with recirculation of nitrate rich liquor from a separate nitrification reactor. Because of loss of reducing power during the nitrification phase, these systems in general require the input of an external carbon source, such as methanol, to ensure that denitrification is complete.

Nanassi *et al* [106] employed a design with pre-denitrification using predominantly native COD (side stream of wastewater) with a high denitrification phase rate (3.6 mM NO_3^- h^{-1}) that is comparable to rates observed in the separated multistage system. The

design of this pre-denitrification approach coupled to the nitrification system was similar to that studied here. However, with no storage used to conserve as much as possible of the native carbon, a high feed C/N ratio was needed to allow for successful denitrification and it was estimated that this system achieved required 17 kg COD/kg N removed with only 70% removal of influent nitrogen. For higher amounts of nitrogen removed, methanol needed to be added into the denitrification reactor.

Table 5.2 Comparison of the PND system developed in this study with similar published separated biomass systems.

Study	Feed C/N (kg COD/kg N)	C/N ratio of removal (kg COD/kg N)
This study	3.7	3.4
Ninassi [106]	NA	17
Rogalla [107]	4.9[17]	>10
Fdez-Polanco [68]	5.8	6.7
Jimenez [108]	7.7	14
Lacamp [8]	NA	2.4[18]
Rodgers [109]	NA	12 – 16
Pedros [110]	10	6.7

A popular design for multistage fixed film reactors appears to be the combination of nitrification and COD removal to limit oxygen transfer to only those processes that require it [108]. The consequence of this is that complete denitrification requires the addition of external carbon thus increasing cost. A particular example of this type of system designed by Rogalla *et al* [107] required C/N ratios in excess of 10 kg COD/kg N-NH_3 for complete nitrogen removal.

[17] Ratio is expressed as BOD/TKN.
[18] See 17.

5.3.4 Effect of a nitrate shock load on denitrification biofilm after continual operation under PND conditions

A test was performed to assess the ability of the denitrification biomass after long term operation in the PND reactor to treat a shock load (6 mM) of nitrate (Figure 5.9) at the beginning of a normal PND phase. Nitrate reduction was rapid (> 3 mM h^{-1}) with first order kinetics and a rate constant of –2.07 h^{-1}. The additional nitrate lowered the C/N feed ratio to 1.7 kg COD/kg N, which in theory does not provide sufficient reducing power for complete denitrification (Appendix A), yet all nitrogen was removed at the end of the cycle (Figure 5.9). If incomplete denitrification to nitric or nitrous oxide is excluded as a likely explanation, this finding may be due to the use of prior stored organic material such PHB oxidation or anoxic maintenance respiration using prior established biomass as electron donor

The nitrate knee routinely observed in literature [81] related to the end of denitrification was not observed in either PND or multistage cycles because the PND system, in which denitrification was coupled to nitrification and the multistage system, allowed the low transfer of oxygen that masked it. The pH increased, as would be expected from the consumption of protons during denitrification.

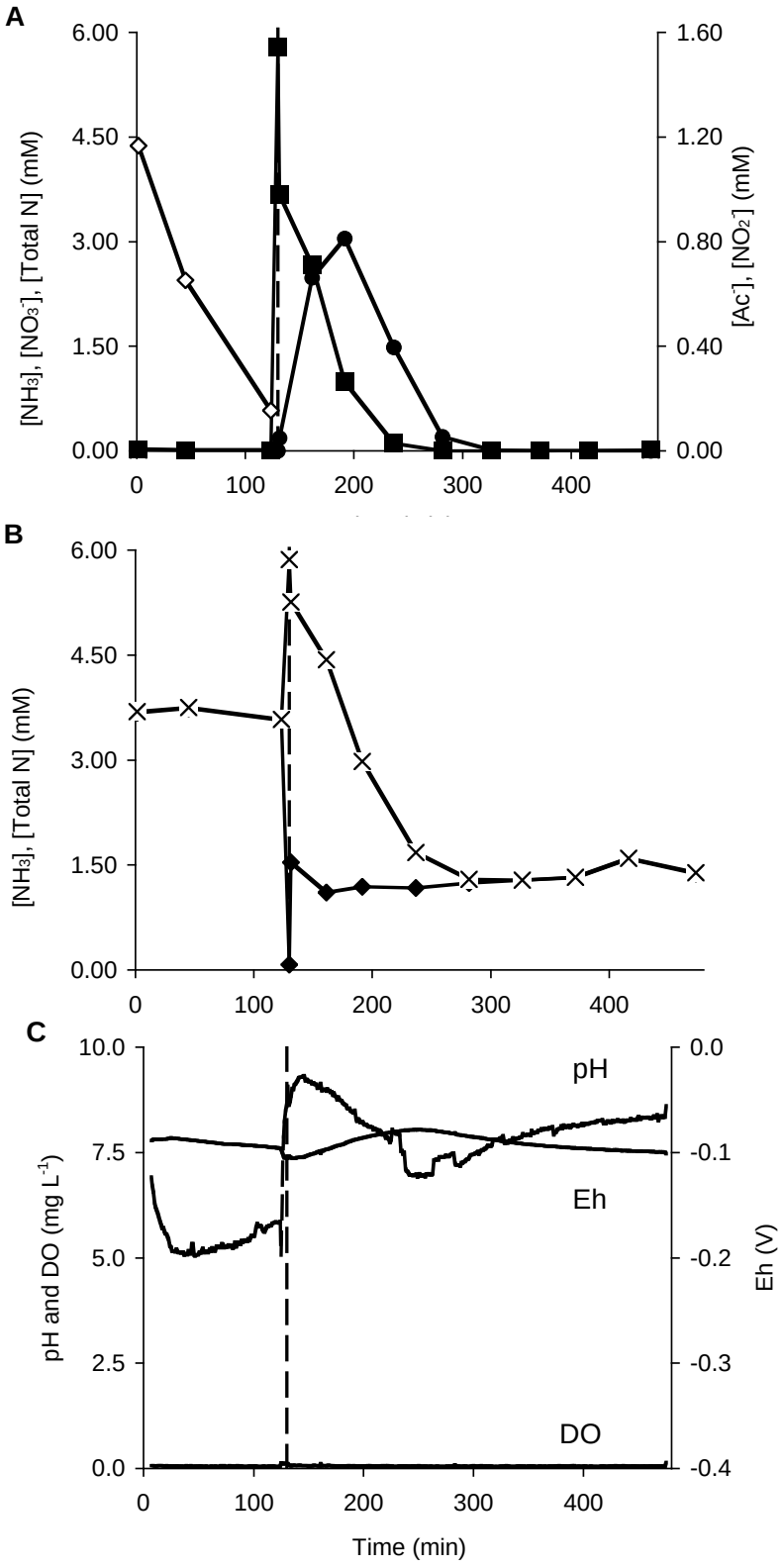

Figure 5.9 Effect of a shock load of nitrate to the storage driven denitrification biofilm reactor after 2 months of continuous PND operation. Nitrate, nitrite, acetate (A), ammonia, total N (B), Eh, pH and DO (C): Acetate (◇), ammonia (◆), nitrate (■), nitrite (●) and total nitrogen (×). Vertical line indicates change in phase from acetate uptake (130 minutes) to denitrification phase (350 minutes).

5.3.5 Luxury storage of COD

In the experiment above, a very low carbon requirement was observed (1.7 kg COD/kg N) which was not due to a substantial short circuit in the denitrification pathway. It was suggested that during preceding cycles, a small proportion of acetate in excess to that required for all activities by the denitrification biomass, was stored in each cycle. After long term operation, it is therefore likely that a substantial store of "luxury" COD would have built up and been available for those cycles where insufficient COD was supplied. The SDDN, and subsequent PND, reactors were fed a fairly constant COD/N feed for a period in excess of three years. Any luxury storage observed in the reactor was the result of ongoing, minimal oversupply of COD and not the result of large and infrequent additions of COD. The fact that luxury storage occurred under limited COD/N supply indicates that the long term performance of the SDDN and PND reactors was good and could have been evaluated more favourably if the "waste" of COD for luxury storage had not occurred. The confirmation that this had occurred in the PND reactor, and the possible extent of luxury storage, need to be assessed.

After a normal acetate uptake phase, the denitrification biofilm was exposed to three successive shock loads (5 mM) of nitrate in order to assess whether and how much luxury storage of COD had occurred over time (Figure 5.10). Nitrate was reduced in each of the successive nitrate fed cycles but with a reduction in denitrification rate overall, which may indicate that the intracellular store of luxury COD was being exhausted (Table 5.3). This indicates that a substantial amount of stored carbon was available to supplement that supplied in the feed during the acetate uptake phase.

PHB and glycogen analysis of the solid samples from this experiment indicated a very high level of polymeric organic carbon present in the biofilm (~20% glycogen and ~2% PHB by mass) (Figure 5.10*C*). While the variation of PHB and glycogen content of the solid samples with time were not clear due to the difficulty of obtaining representative samples, the average amount of stored carbon indicates that even at the low C/N ratio feeds used in this study an oversupply of carbon had routinely been given to the culture.

Table 5.3 Rate of nitrate reduction during sequential repeated shock loads of nitrate to the PND system after continuous operation for 2 months.

Cycle number	Initial [NO_3^-] (mM)	Rate of nitrate removal	
		(mM N h^{-1})	(mM N h^{-1} bioball^{-1})
1	5.15	2.16	4.5×10^{-3}
2	4.58	1.86	4.0×10^{-3}
3	5.14	1.60	3.5×10^{-3}

The extracellular polymeric substances (EPS) material in which biofilms reside vary with individual biofilms. It is possible that the very high amount of glycogen was an increasingly important component of the EPS material that bound the denitrification biofilm together. Polysaccharides have been observed in very large amounts in EPS in other systems [111] and polysaccharide material in the EPS can be utilised under conditions of starvation [112].

In each cycle, the variation of the redox potential with time was observed to show a similar pattern. However, the absolute values of redox potential observed increased in each successive cycle (Figure 5.10*B*). As more of the intracellular material was consumed, this reflected a general decrease in the reduced nature of the biomass.

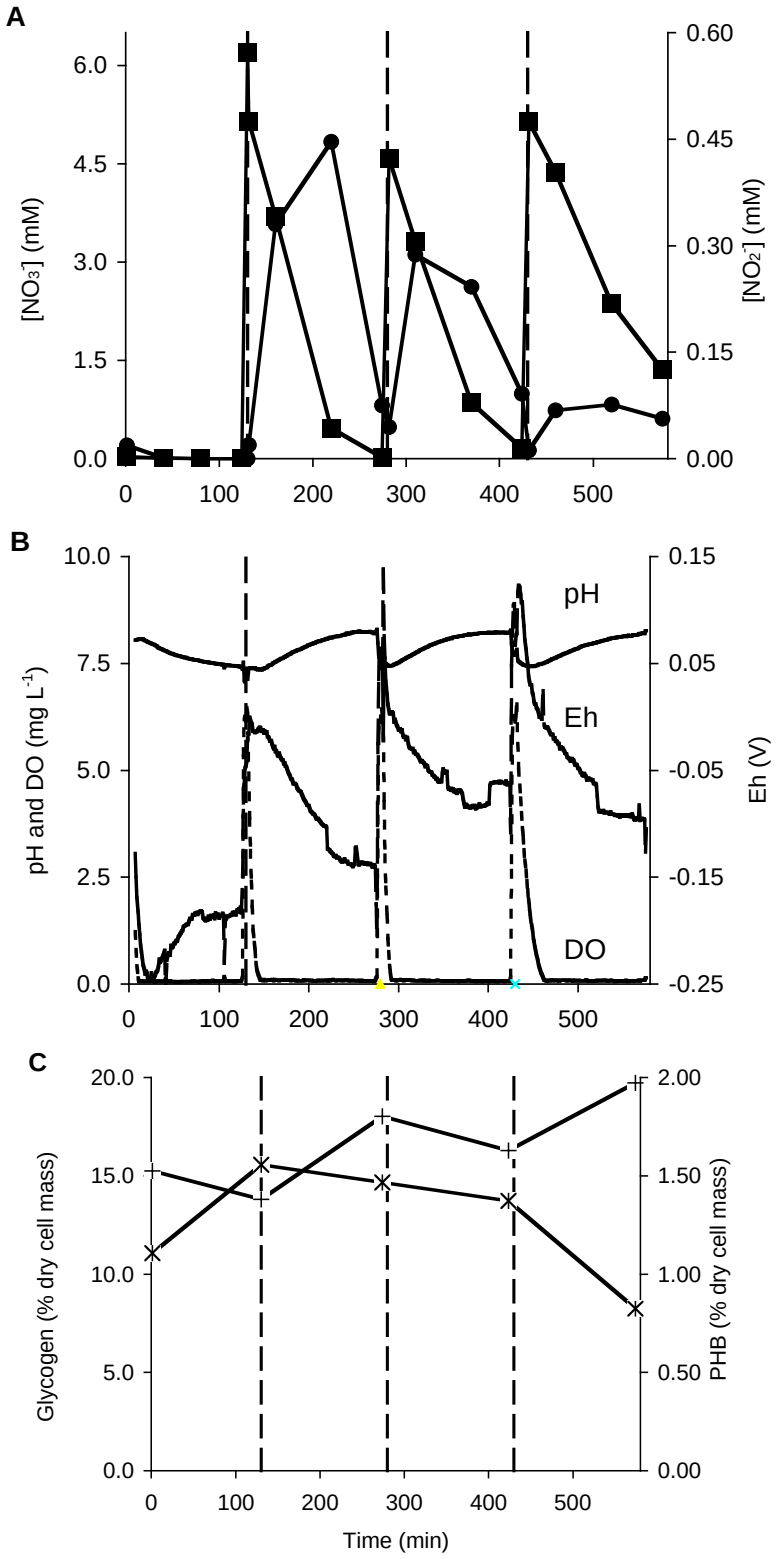

Figure 5.10 Effect of a repeated shock load of nitrate to the storage driven denitrification biofilm reactor after 2 months of continuous PND operation. Nitrate, nitrite (A), Eh, pH and DO (B) and PHB and glycogen (C): Nitrate (■), nitrite (●), acetate (◇), PHB (×) and glycogen (+). Vertical lines indicate phase changes.

Effect of luxury storage on nitrogen removal in the PND reactor

From the results of the nitrate shock load test it was clear that more COD was available to the culture than that formally delivered in the feed. From these results, and the oxygen control tests discussed in Chapter 6, it would appear that a reasonable amount of stored COD was available to the culture when required. This was not the case in the past when the amount of nitrogen removal was directly limited by the amount of COD supplied (Section 2.3.3).

To quantify the amount of luxury storage that may have occurred over 6 months of continuous operation as a PND process, the culture was fed a no COD feed with normal ammonia (Figure 5.11). The removal of 79% of influent nitrogen was observed. This confirms that some level of luxury storage had occurred under PND operating conditions with the relatively low feed ratio of 3.7 kg COD/kg N, compared to the local Woodman Point WWTP of 11 kg COD/kg N. Any further tests performed to specifically test the C/N treatment ratio possible would need to limit the C/N ratio of the feed further for a sustained period of time.

5.3.6 Treatment of real wastewater by the PND reactor

The very clean carbon and nitrogen sources that make up the synthetic feed and therefore dictate the possible storage mechanism of carbon may mean that, although the performance of the PND reactor is promising, it is still a laboratory based system that may in fact not work without the very defined feed from the laboratory. A better test of the performance of the system, for use as an applied wastewater treatment process,

would be to give the bacteria a real wastewater feed for multiple cycles and test the performance.

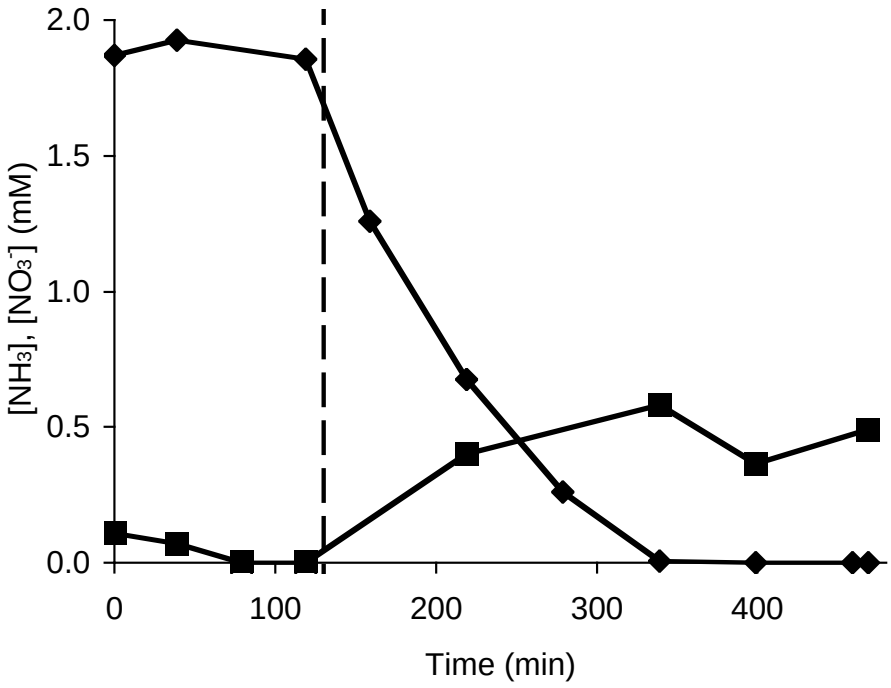

Figure 5.11 Test of capacity of PND reactor to treat no COD wastewaters due to accumulated intracellular COD from preceding cycles. Ammonia (◆) and nitrate (■). Vertical line indicates change in phase from acetate uptake (130 minutes) to nitrogen removal phase (350 minutes).

A real wastewater was obtained from a local wastewater treatment plant post primary treatment as an appropriate feed for use in this system (Section 2.2.1). Four cycles were operated with this feed, with the first and fourth monitored for nitrogen removal activity (Figures 5.12 and 5.13). The two monitored cycles show nearly identical behaviour to each other and to the general pattern of PND treatment routinely observed.

Measured soluble COD was substantially removed during the cycle (from 590 mg L^{-1} to 170 mg L^{-1}). Literature observation of biofilm use in wastewater treatment indicates that biofilms can concurrently act as filtration devices as well as biocatalysts for nutrient

removal [113] which can account for large amounts of COD removal even if not converted to PHB or biomass. In contrast, biofilms can be modified by the adsorption of suspended solids [114], which would be expected to change the long term performance of the system. In examples where suspended solids negatively affect the performance of a biofilm, pre-treatment such as filtering through a sand bath could be used to alleviate the problem. The influent C/N ratio to the system was 9.4 kg COD/kg N and resulted in the removal performance where 7.6 kg COD were consumed for every kilogram N of nitrogen removed.

Despite the real wastewater being high in COD, it was quite low in acetate (< 1.3 mM). Gas chromatography analysis showed that besides acetate, no other VFAs such as propionate or butyrate were present at significant concentrations (acetate is routinely >80% of total VFA content). The identity of soluble COD in the real wastewater is not known explicitly and is likely to be variable. The supply of COD, regardless of form, was able to be used for nutrient removal by the PND system. The possibility of denitrification being driven by stored material from previous cycles with synthetic wastewater could be excluded as the real wastewater nitrogen removal also occurred after 4 cycles with real wastewater.

The ammonia in both cycles decreased linearly as observed in previous PND cycles at a rate of 0.53 mM h^{-1} overall. As with the normal PND synthetic waste fed operation, there was minimal accumulation of nitrate and nitrite. The ability of the PND reactor to adequately treat a real rather than synthetic wastewater indicates that although the mixed COD sources in a real wastewater would alter the metabolic processes involved in storage, sequestration of COD still occurred and was available for nutrient removal. As the performance of the PND system in terms of C/N ratio of removal is very

competitive, it is probably best applied in the treatment of those wastewaters with very low C/N ratios, unlike urban municipal wastewater.

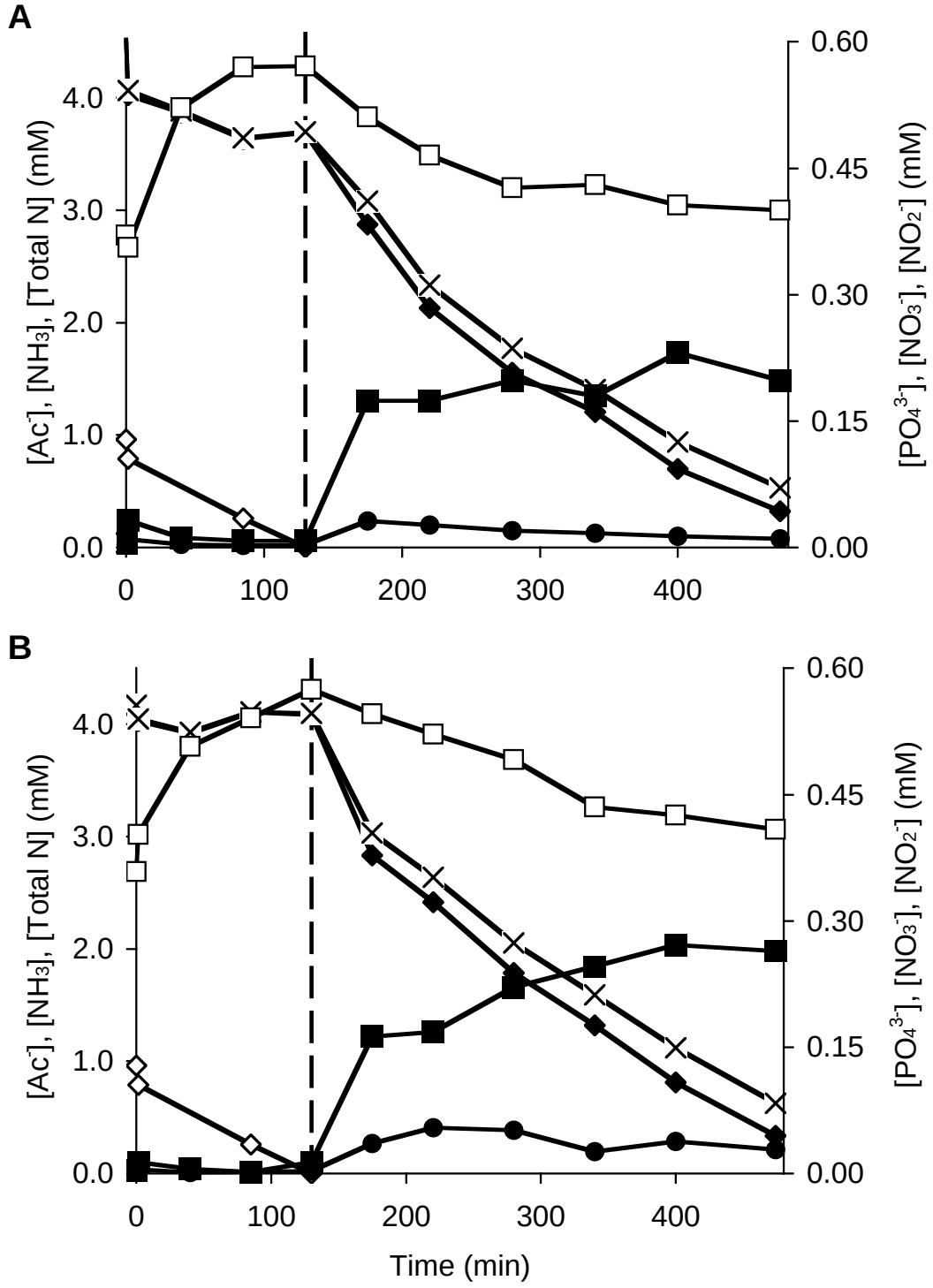

Figure 5.12 Test of real wastewater on PND system after long term operation with synthetic wastewater observed during the first (A; 1.0 kg COD/kg N-NH_3 based on acetate content only) and fourth (B; 1.1 kg COD/kg N-NH_3 based on acetate content only) of four sequential cycles: Acetate (◇), phosphate (□), ammonia (◆), nitrate (■), nitrite (●) and total nitrogen (×).Vertical line indicates change in phase from acetate uptake (130 minutes) to nitrogen removal phase (350 minutes).

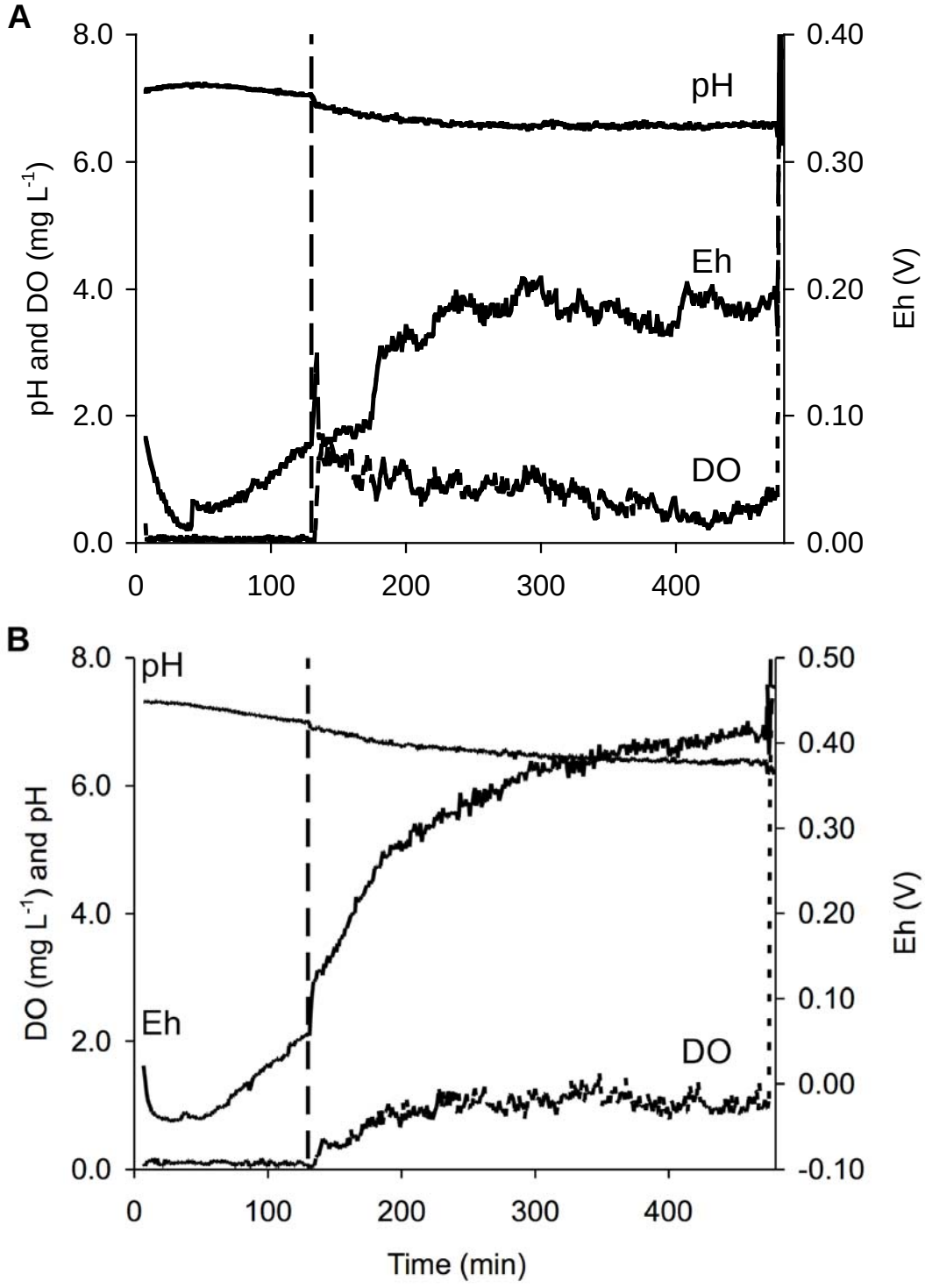

Figure 5.13 Reproducibility of DO, Eh and pH during the first (A; 1.0 kg COD/kg N-NH_3) and fourth (B; 1.1 kg COD/kg N-NH_3) of four sequential cycles of PND operation with a real wastewater as substrate. Vertical line indicates change in phase from acetate uptake (130 minutes) to nitrogen removal phase (350 minutes).

5.3.7 Phosphate metabolism in the PND reactor

Phosphate metabolism under PND conditions appeared to change with time from that observed under storage driven denitrification or multistage conditions. The change not only coincided with the switch to PND operation but also to a lengthy period of storage at 4°C of the storage driven denitrification biofilm (Section 7.3.6).

Consider the phosphate metabolism that was observed during storage driven denitrification operation and maintained when connected in a multistage system to a suitable nitrification culture. Phosphate metabolism may in fact have represented the possibility of using the multistage system as a simultaneous nitrogen and phosphorus removal process akin to systems like Dephanox [48, 49, 87] and alternating pumped sequencing batch biofilm reactor (APSBBR) [109, 115].

Over time, it would appear that the ability to absorb and release phosphate has diminished (Figure 5.12). This is most likely to be due to the loss of bulk electron acceptor availability to the denitrification culture as a result of cycling reactor liquor through the nitrification reactor simultaneously. Nitrate concentration throughout the nitrogen removal phase under PND conditions was usually less than 0.5 mM, which may be insufficient to encourage anoxic uptake of phosphate. This system, as discussed in Chapter 1, was always principally focussed on nitrogen and not phosphorus removal but there was interest generated by the possibility that it could in fact do simultaneous N and P removal. This may not be the case if the system is operated in parallel rather than sequential multistage operation.

The change in phosphate metabolism is unlikely to be an effect caused by the renewal and dilution of phosphate from the system as the nitrifiers did not metabolise phosphate (Figure 4.14). Nor is it likely to be an effect of greater oxygen in the denitrification phase since the bulk of the experiments shown are at very low operating DO (<0.25 mg L^{-1}) during PND and multistage operation, and therefore allow minimal transfer of oxygen to the denitrifiers. The most likely cause is that the denitrification reactor was supplied minimal nitrate and was therefore operating close to anaerobic conditions under which phosphate accumulation would be limited.

5.4 Conclusions

Under PND operation, nitrogen removal occurs at high rates where both nitrification and denitrification occur simultaneously. The rate of nitrogen removal was limited by the rate of nitrification and hence can be increased by an increase in the nitrifying biomass present. The carbon requirement for nitrogen removal was very low, frequently below the theoretical stoichiometric minimum – likely to be the result of luxury storage of COD from previous cycles. The PND reactor was also able to achieve complete nitrogen removal using the complex COD input found in a real municipal wastewater.

Chapter 6. Effect of oxygen on the long term performance of PND for nitrogen and COD removal

Abstract

The dependence of the previously described parallel nitrification and denitrification (PND) process on oxygen supply was investigated. The PND process involves a COD storage phase by a heterotrophic denitrification biofilm followed by a nitrogen removal phase. During the nitrogen removal phase, the ammonia rich wastewater was circulated continuously between physically separated biofilms performing nitrification and denitrification. Under conditions with very limited oxygen supply ($k_La << 1\ h^{-1}$; feed 3.7 kg COD/kg $N\text{-}NH_3$), ammonia oxidation was significantly decreased from 100% to below 30%. The oxidation of ammonia was observed to be the rate limiting stage of the PND process. Under conditions of elevated dissolved oxygen (up to 4 mg L^{-1}), the PND reactor continued to perform competitively with respect to overall rate of nitrogen removal (0.45 mM h^{-1}) and carbon requirement for complete nitrogen removal (< 2.9 kg COD/kg $N\text{-}NH_3$). The very low carbon requirement may be the result of a short circuit between nitrification and denitrification via nitrite or the result of luxury storage of COD in previous cycles. Denitrification was negligibly affected by elevated dissolved oxygen. The robustness of the PND reactor with respect to tolerance to dissolved oxygen makes the PND reactor superior to SND based systems where sophisticated dissolved oxygen control is critical for efficient nitrogen removal.

6.1 Introduction

The control of oxygen is an important parameter in the achievement of nitrogen removal [116]. A careful balance must be sought between sufficient supply for efficient ammonia oxidation by nitrifying organisms while minimising inhibition of denitrification activity [29, 116]. Nitrogen removal from wastewater under virtually any configuration requires this balance to be achieved, directly or indirectly. In particular, under SND operation, where both nitrification and denitrification processes occur simultaneously, the delivery of oxygen must be carefully controlled to allow both processes to occur at appreciable rates [29]. The actual supply of oxygen required in SND systems will depend, for example, on the size and structure of the microbial floc or biofilm involved [15]. A dense or large floc, may predominantly allow growth of aerobic nitrifiers in the outer layers of a floc while the internal areas are dominated by denitrifying organisms due to the chemical gradient of oxygen distribution throughout the floc [41, 117].

The challenge in oxygen supply is to limit wastage of COD by aerobic respiration so that DO is available for ammonia oxidation and COD available for denitrification [54]. The PND reactor has similar requirements. A better understanding of the long term development of the PND culture and the consequences of oxygen control on the performance of the PND system was sought. As DO is a critical operational parameter for SND systems, an investigation was undertaken to assess whether it was also critical for the PND reactor and if so, at what DO was nitrogen removal optimal.

6.2 Materials and Methods

6.2.1 Laboratory reactor design and operation

All configurations of the PND and storage driven denitrification reactors used in this chapter employed the same acetate uptake phase as the storage driven denitrification, multistage and PND reactors in previous chapters. The reactor design used for the work in this chapter was identical to that described in Chapter 5 (Section 5.2.1). The lengths of the two phases in the trials are detailed in the results and discussion section where appropriate but, as in all previous configurations, the fill and decant phases were rapid (< 3% total cycle time). To test the effect of oxygen on denitrification activity, the storage driven denitrification reactor (Section 2.2.2) was reinstated.

A variety of different oxygen delivery methods were tested in this study. For tests under oxygen limitation, a bubble diffuser supplied with nitrogen was introduced to the recycle vessel to maintain a negligible dissolved oxygen level in the reactor liquor. Oxygen delivery under these conditions was achieved by diffusion of air into the open headspace of the nitrification chamber. At high operating oxygen concentrations, oxygen was delivered at a higher rate to the recycle vessel *via* a bubble diffuser. Specific k_La values are given with each result.

The feed supplied to the reactor was the same as that used for the multistage system (Section 4.2.1) unless otherwise specified in the results and discussion. Where changes were necessary, this was only in terms of the C/N ratio of the influent feed.

6.2.2 Analysis

All nitrogen and phosphorus compounds were measured spectrophotemetrically as described in Chapter 2 (Section 2.2.4). The acetate and PHB content of lyophilised biomass was measured by gas chromatography (Section 2.2.4).

6.2.3 Calculations

Measurement of k_La, calculation of OUR and hence oxygen consumed, C/N treatment ratios and overall rates of nitrogen and ammonia removal were performed as described previously (Section 4.2.4).

6.3 Results and Discussion

6.3.1 Effect of low oxygen supply

As discussed previously, a major challenge with biological nitrogen removal using either simultaneous or parallel nitrification and denitrification from wastewater is the efficient supply and use of oxygen to allow for nitrification but not inhibit denitrification nor waste COD. The direction of flow of liquor through the reactor passes from the recycle vessel to the denitrification chamber, the nitrification chamber and back to the recycle vessel (Figure 5.1). Due to the expected inhibition of denitrification by oxygen, it was thought necessary to substantially reduce the oxygen

supply to the nitrification column and to strip residual oxygen from the recycle vessel to ensure that transfer of oxygen to the denitrification biofilm was negligible. Therefore, in tests under very limited oxygen supply, the recycle vessel liquor was continuously degassed with nitrogen to remove residual oxygen before the liquor entered the denitrification reactor (Section 6.2.1). This had the effect of also reducing the supply of oxygen to the nitrification biofilm due to the net outflow of nitrogen from the reactor preventing the inflow of air to that reactor. The resulting oxygen mass transport coefficient was very low ($k_La << 1\ h^{-1}$; average dissolved oxygen < 0.4 mg L^{-1}).

As expected, the very low supply of oxygen ($k_La << 1.0\ h^{-1}$; average DO = 0.15 mg L^{-1}, average OUR < 8 mg L h^{-1}) during the nitrogen removal phase limited nitrogen removal by interfering with nitrification. The OUR observed would limit the removal of ammonia to less than 1.6 mM ($k_La << 1.0\ h^{-1}$, average OUR < 8 mg L h^{-1}, PND cycle length 3 hours, ∴ maximum amount of oxygen consumed 24 mg L^{-1} or 0.8 mM and hence maximum ammonia consumed will be 1.6 mM). The observed results are in line with those predicted. Only 22% of ammonia was removed (Figure 6.1). Nearly all (80%) of the ammonia lost was removed completely *via* denitrification, as would be expected under limited oxygen supply. This outcome was consistent with repetitive testing (Figure 6.2). The ongoing limited supply of oxygen resulted in the loss of nitrification activity over the course of 15 days (Figure 6.3). It is unclear whether this was a result of loss of viability of the biomass as the nitrification biofilm was reinoculated in an attempt to quickly increase activity, although it is likely based on literature evidence for the absolute requirement for oxygen by nitrifiers (Section 1.2.1).

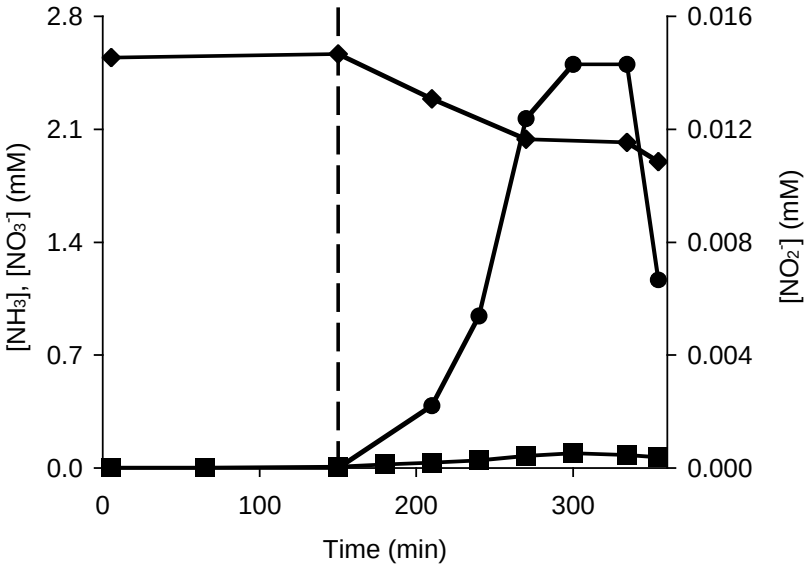

Figure 6.1 Effect of very low oxygen supply ($k_La << 1\ h^{-1}$ and average operational DO 0.15 mg L^{-1}) on nitrogen removal by the newly established PND reactor. Feed: Ammonia 2.5 mM and Acetate 2.1 mM; 3.7 kg COD/kg N-NH_3. Ammonia (◆), nitrate (■) and nitrite (●). Vertical line indicates phase change from acetate uptake phase (150 minutes) to PND phase (210 minutes).

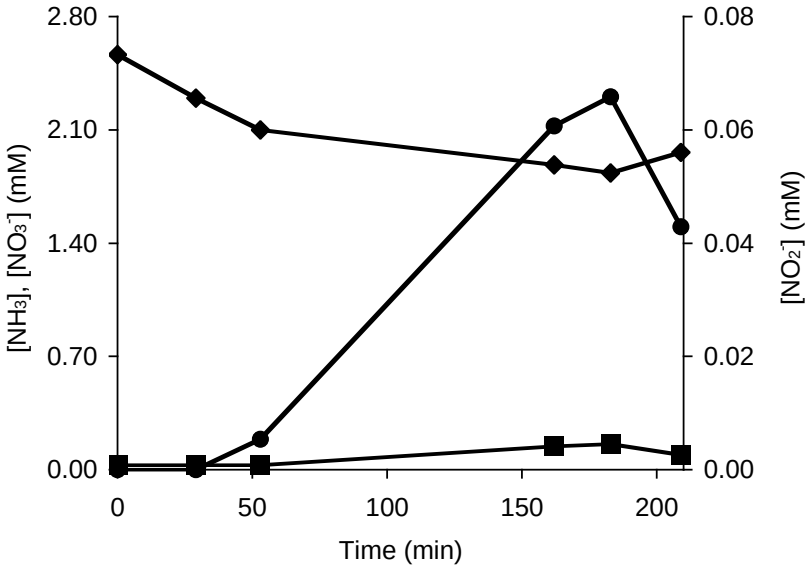

Figure 6.2 Reproducible effect of very low oxygen supply ($k_La << 1\ h^{-1}$ and average operational DO 0.38 mg L^{-1}) on nitrogen removal by the newly established PND reactor during the PND phase (210 minutes). Feed: Ammonia 2.6 mM and Acetate 2.1 mM; 3.7 kg COD/kg N-NH_3. Ammonia (◆), nitrate (■) and nitrite (●).

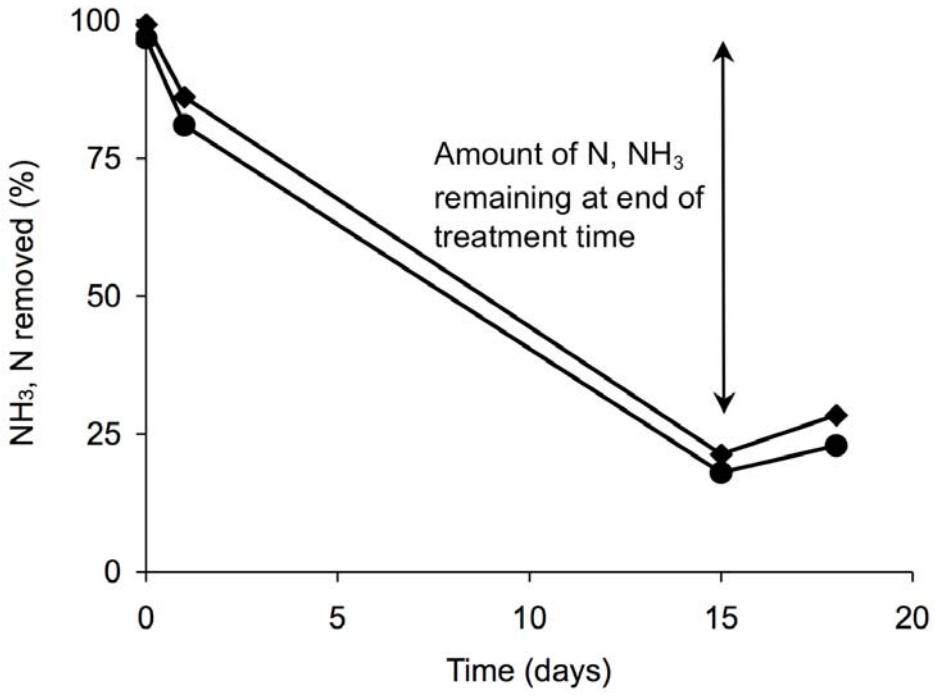

Figure 6.3 Prolonged effect of long term low oxygen supply ($k_La << 1\ h^{-1}$) on the nitrogen removal of the PND reactor. Feed: Ammonia 2.6 mM and Acetate 2.1 mM; 3.7 kg COD/kg N-NH_3. Ammonia (◆), total nitrogen (●). Acetate uptake phase length 150 minutes and PND phase length > 190 minutes.

6.3.2 Effect of high dissolved oxygen on the established PND culture

After a relatively long period (6 months) of operation under controlled PND conditions, where nitrogen removal occurred at controlled dissolved oxygen by the combined, parallel effort of separated nitrification and denitrification biofilms, the effect of high dissolved oxygen concentration during a cycle was tested. It was expected that a higher oxygen supply would affect the system by preventing storage as has been observed in SND systems [37, 116]. Each of the three metabolic activities involved in PND, acetate storage, nitrification, denitrification, was tested at high (> 2 mg L^{-1}) dissolved oxygen.

Acetate uptake with high dissolved oxygen supplied

The kinetics of acetate uptake were not affected by the increase in DO as the behaviour was identical to trials under low oxygen conditions (Section 2.3.1 and 5.3.1), with apparent first order kinetics (Figure 6.4). It was expected that a reduced amount of storage would occur of the influent acetate as a result of the increased oxygen supply as has been observed by other workers [18, 28, 37].

The uptake of acetate under the higher dissolved oxygen conditions (k_La 7.8 h^{-1}; average DO 4.5 mg L^{-1}) resulted in an increase in PHB in the biomass (Figure 6.4). Under oxygen limiting conditions ($k_La << 1$ h^{-1}; average DO 0.45 mg L^{-1}) of the subsequent denitrification phase, all nitrate was removed (Figure 6.4) indicating sufficient storage was available. The drop of PHB to the level prior to acetate uptake (Figure 6.4) suggests that even at high dissolved oxygen, storage of influent COD continued. Analysis of the oxygen consumed during acetate uptake reveals that under the elevated dissolved oxygen supplied, up to 56% (average OUR = 26 mg L^{-1} h^{-1}, amount of oxygen consumed = 57 mg L^{-1} or 1.8 mM, $\therefore$ 0.9 mM of fed 1.6 mM acetate was oxidised) of the influent acetate should have been oxidised. It was expected that the remaining acetate (44%) was stored. This effect of oxygen was reported by Third *et al* [37], who observed that under oxygen limiting conditions (k_La = 6.0 h^{-1}; DO = 0 mg L^{-1}), 63% of influent acetate was stored as PHB while at higher oxygen delivery conditions (k_La = 51 h^{-1}; DO > 0.9 mg L^{-1}) this was reduced to 48% of influent acetate. It should be noted that Third *et al* [37] were using a suspended culture SBR and hence had considerably lower biomass than in this study (approximately 10 fold; Appendix F).

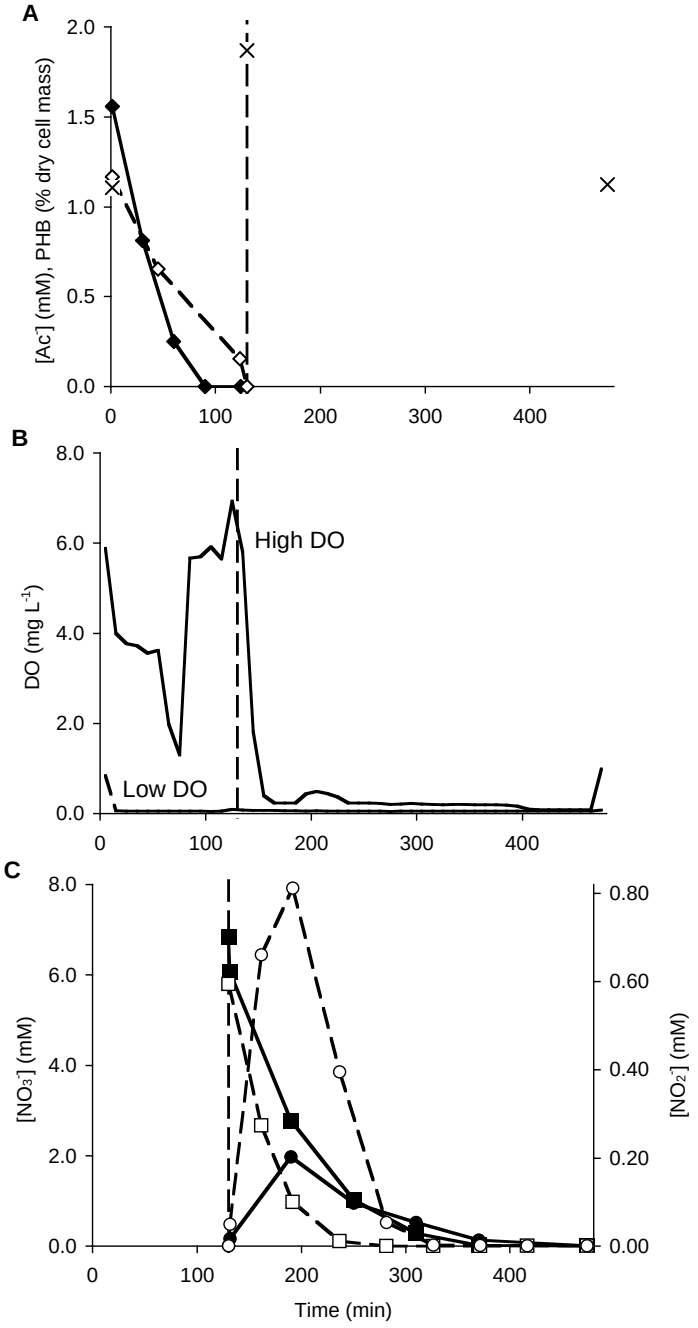

Figure 6.4 Acetate uptake under elevated oxygen supply conditions (k_La 7.8 h^{-1}, average DO 4.5 mg L^{-1}) in the storage driven denitrification reactor. Normal oxygen supply conditions (k_La << 1 h^{-1}, average DO 0.12 mg L^{-1}) shown for comparison. High DO: Acetate (◆), PHB (×), nitrate (■) and nitrite (●); Normal DO: Acetate (◇), nitrate (□) and nitrite (○). Vertical line indicates phase change from acetate uptake phase (130 minutes) to denitrification phase (350 minutes)[19].

[19] Denitrification phase operated under low oxygen supply conditions (k_La << 1 h^{-1}, average DO 0.43 mg L^{-1}) as normal (Section 2.2.2). For high DO experiment: feed acetate uptake phase: Ammonia 3.6 mM and Acetate 1.6 mM. Feed denitrification phase: Nitrate 6.8 mM. Overall feed ratio 1.0 kg COD/kg N-NO_3^-. For normal DO experiment: feed acetate uptake phase: Ammonia 3.7 mM and Acetate 1.2 mM. Feed denitrification phase: Nitrate 5.8 mM. Overall feed ratio 0.92 kg COD/kg N-NO_3^-.

The reduction in stored amount of influent acetate should have an effect on the amount of nitrogen removal achieved in the subsequent denitrification phase. However, much more nitrate was removed than expected (Figure 6.4). Where only 44% (0.7 mM) of the influent acetate was available for storage, only 1.1 mM of nitrate should have been treated to completion. The level of treatment of nitrate observed was considerably higher (Figure 6.4, 5.2 mM total N removed). This suggests that more stored material was available than that produced in the acetate uptake phase immediately preceding denitrification, as observed earlier (Section 5.3.5).

Denitrification at high dissolved oxygen

Under high oxygen concentration, denitrification in the storage driven denitrification reactor occurred with similar kinetics as that under low oxygen storage driven denitrification conditions (Figure 6.5). While the supply of acetate was low (1.5 mM) compared to the nitrate supplied (6.0 mM) during the denitrification phase, nitrate was still substantially removed with a very low carbon requirement for observed nitrogen removal (1.1 kg COD/kg $N\text{-}NO_3^-$). The average OUR observed during denitrification should have caused the loss of all of the supplied electrons from the acetate uptake phase (average OUR = 30 mg L^{-1} h^{-1}, amount of oxygen consumed = 176 mg L^{-1} or 5.5 mM, $\therefore$ the equivalent electrons from 2.7 mM of fed 1.5 mM acetate should have been oxidised). This further supports the conclusion that substantial luxury storage of superfluous COD had accumulated (Section 5.3.5). Under high oxygen conditions, however, it would be expected that, regardless of COD supply, denitrification would be inhibited (Section 1.2.2). Denitrifying organisms tend to be facultative with respect to the use of terminal electron acceptors, with oxygen preferentially used. That is, under

conditions where oxygen is supplied, these organisms tend to use oxygen rather than nitrate even if nitrate is available in abundance [14, 15, 17].

A simple model, conservatively based on the rate of denitrification would cause at least 15% of the reactor to be free of dissolved oxygen (Appendix G) assuming a concentration gradient due to approximately plug flow conditions [80, 118]. Diffusion limitation of oxygen through the biofilm was likely to have increased this percentage [119, 120]. As a result, it was likely that anoxic conditions, conducive to denitrification, were established in a substantial proportion of the biofilm.

Very well studied microorganisms capable of denitrification were consistently observed to preferentially use oxygen in place of nitrate where both were supplied [47]. Early reports of denitrification under aerobic conditions were dismissed due to either lack of measurement or control of oxygen or heterogeneity in the structure of the biofilms or flocs involved that could allow for microanaerobic environments to develop [47]. *Paracoccus denitrificans* (formerly *Thiosphaera pantotropha* [45]), a denitrifying mixotroph, was able to co respire both oxygen and nitrate as electron acceptors even up to 90% of air saturation [47]. Constitutive nitrate reductases have since been observed in a variety of bacteria [47, 121]. While literature suggest that aerobic denitrification may be part of the mechanism of PND, because of plug flow conditions and biofilm growth, aerobic denitrification is not likely or necessary to explain the results in this study.

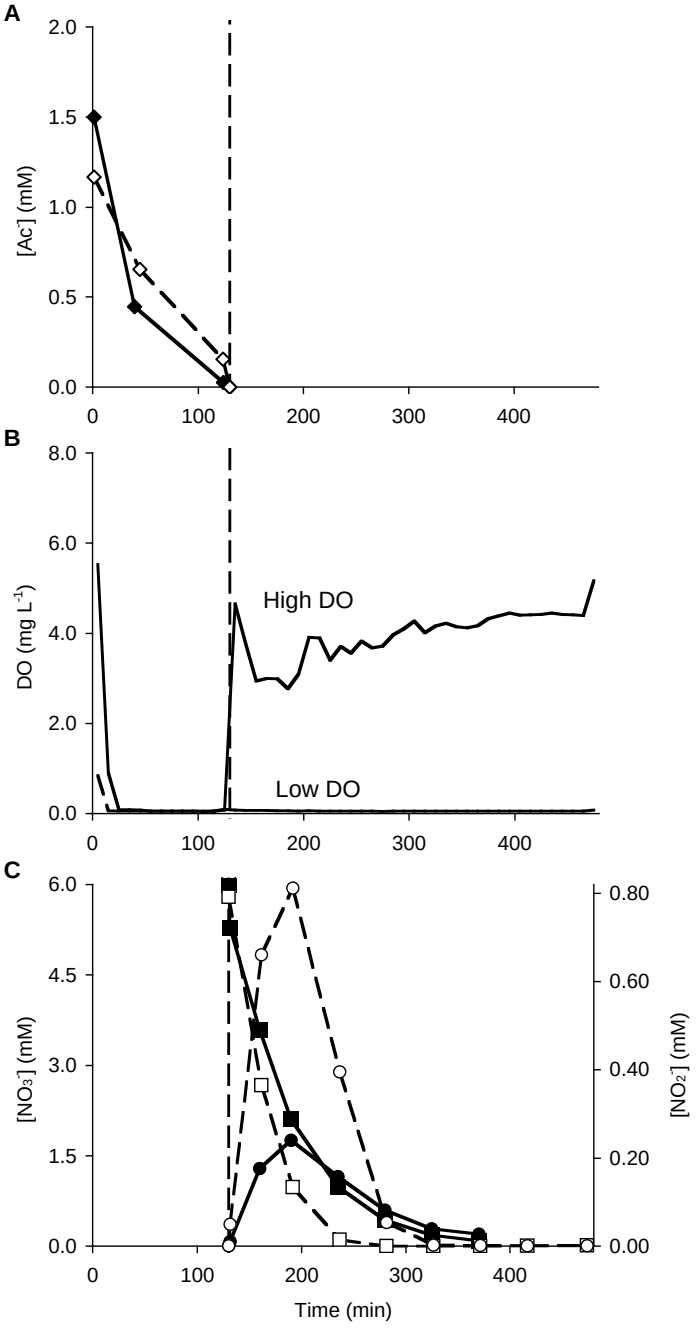

Figure 6.5 Denitrification under elevated oxygen supply conditions (k_La 7.8 h^{-1}, average DO 4.0 mg L^{-1}) in the storage driven denitrification reactor. Normal oxygen supply conditions (k_La << 1 h^{-1}, average DO 0.06 mg L^{-1}) shown for comparison. High DO: Acetate (◆), nitrate (■) and nitrite (●); Normal DO: Acetate (◇), nitrate (□) and nitrite (○). Vertical line indicates phase change from acetate uptake phase (130 minutes) to denitrification phase (350 minutes)[20].

[20] Acetate uptake phase operated under low oxygen supply conditions (k_La << 1 h^{-1}, average DO 0.57 mg L^{-1}) as normal (Section 2.2.2). For high DO experiment: feed acetate uptake phase: Ammonia 2.5 mM and Acetate 1.5 mM. Feed denitrification phase: Nitrate 6.0 mM. Overall feed ratio 1.1 kg COD/kg N-NO_3^-. For normal DO experiment: feed acetate uptake phase: Ammonia 3.7 mM and Acetate 1.2 mM. Feed denitrification phase: Nitrate 5.8 mM. Overall feed ratio 0.92 kg COD/kg N-NO_3^-.

Nitrogen removal *via* PND at high dissolved oxygen

The PND phase of the reactor was repeatedly able to remove substantial amounts of nitrogen with relatively low COD supply at high DO (Figures 6.6 and 6.7). The rate of ammonia removal during the PND phase under high oxygen conditions was slightly lower (Figures 6.6 and 6.7; 0.6 mM h^{-1}) than under normal oxygen supply conditions (Figures 6.6 and 6.7; 0.8 mM h^{-1}) indicating that very little inhibition of denitrification occurred under the higher dissolved oxygen. The transient appearance of nitrite in each replicate (Figures 6.6 and 6.7) did not consistently indicate the inhibition of denitrification.

While the COD supply was most likely substantially supplemented by the presence of stored excess COD from previous cycles, and the oxidation of ammonia was as expected, the removal of nitrate and nitrite under the high oxygen conditions was unexpected supporting the conclusion (above) that diffusion limitation of oxygen protected the active denitrifiers in the storage driven denitrification biofilm.

An analogous study of the effect of oxygen concentration on SND performance by Third *et al* [29] revealed an increase in the concentration of dissolved oxygen (0.5 to 5.0 mg L^{-1}) resulted in a faster rate of ammonia oxidation (3.3 to 8.0 mmole NH_3 $CmolX^{-1}$ h^{-1}) but caused a substantial reduction in removal of nitrogen (93 to 44%) as a direct result of the inhibition of denitrifiers. The inhibition of denitrification at high DO has been observed in literature [41, 89, 98, 116]. Earlier results of the PND system demonstrated that elevated DO reduced denitrification activity (Figure 5.3; Section 5.3.1). Long term operation of the PND reactor (> 6 months after Figure 5.3) was likely to increase the thickness of the biofilm and hence the diffusion limited transport of

oxygen to the bacteria in the biofilm resulting in different 'tolerance' to oxygen than observed previously.

6.4 Conclusions

While it is clear that a minimum of oxygen supply is needed to ensure that nitrification activity is maintained, the PND reactor is not sensitive to fluctuations in dissolved oxygen. This represents a clear advantage over SND systems in which the maintenance and control of oxygen supply is critical to activity.

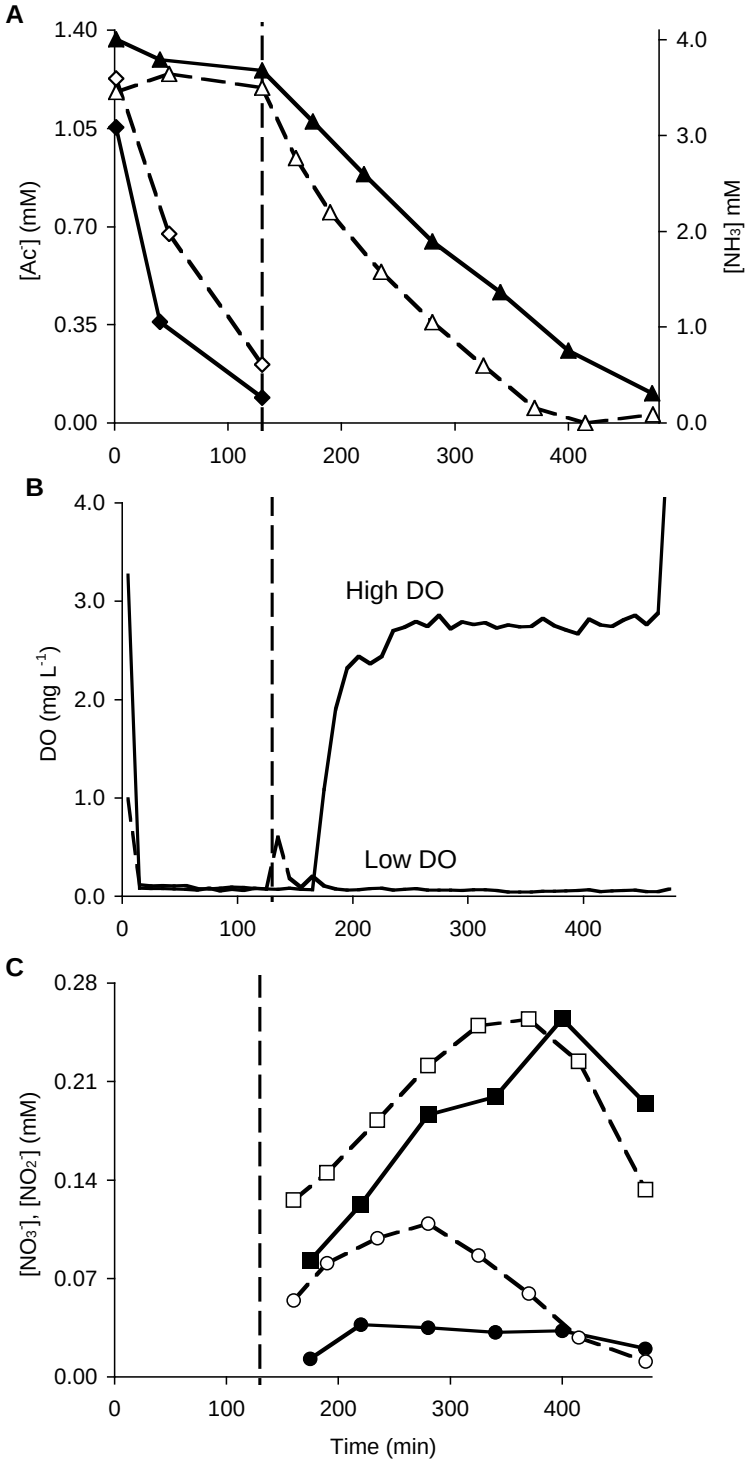

Figure 6.6 Nitrogen removal *via* PND under elevated oxygen supply conditions (k_La 15 h^{-1}, average DO 2.4 mg L^{-1}). Normal oxygen supply conditions (k_La << 1 h^{-1}, average DO 0.08 mg L^{-1}) shown for comparison. High DO: Acetate (◆), ammonia (▲), nitrate (■) and nitrite (●); Normal DO: Acetate (◇), ammonia (△), nitrate (□) and nitrite (○). Vertical line indicates phase change from acetate uptake phase (130 minutes) to PND phase (350 minutes)[21].

[21] Acetate uptake phase operated under low oxygen supply conditions (k_La << 1 h^{-1}, average DO 0.34 mg L^{-1}) as normal (Section 2.2.2). For high DO experiment: feed Ammonia 4.0 mM and Acetate 1.1 mM; feed ratio 1.2 kg COD/kg N-NH_3. For normal DO experiment: feed Ammonia 3.5 mM and Acetate 1.2 mM; feed ratio 1.6 kg COD/kg N-NH_3.

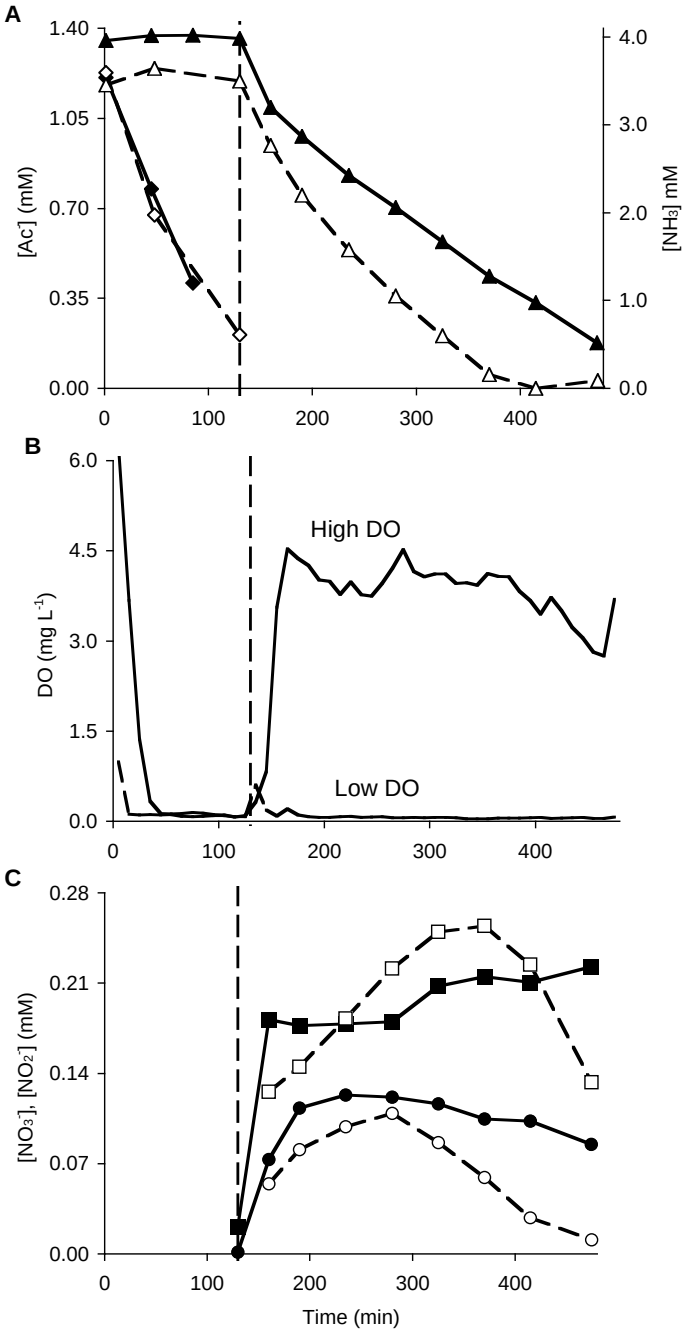

Figure 6.7 Reproducible effect of high oxygen supply conditions (k_La 15 h^{-1}, average DO 2.4 mg L^{-1}) on the removal of nitrogen in the PND reactor during the PND phase. Normal oxygen supply conditions (k_La << 1 h^{-1}, average DO 0.08 mg L^{-1}) shown for comparison. High DO: Acetate (◆), ammonia (▲), nitrate (■) and nitrite (●); Normal DO: Acetate (◇), ammonia (△), nitrate (□) and nitrite (○). Vertical line indicates phase change from acetate uptake phase (130 minutes) to PND phase (350 minutes)[22].

[22] Acetate uptake phase operated under low oxygen supply conditions (k_La << 1 h^{-1}, average DO 0.34 mg L^{-1}) as normal (Section 2.2.2). For high DO experiment: feed Ammonia 4.0 mM and Acetate 1.4 mM; feed ratio 1.2 kg COD/kg N-NH_3. For normal DO experiment: feed Ammonia 3.5 mM and Acetate 1.2 mM; feed ratio 1.6 kg COD/kg N-NH_3.

Chapter 7. Effect of liquor exchange on the performance of the PND system

Abstract

Optimisation of the previously described parallel nitrification and denitrification (PND) reactor was investigated with respect to mechanical control parameters and also to quantify the amount of denitrification activity required to achieve complete nitrogen removal. A range of trials was conducted in which the relative rate of liquor flow in each reactor was manipulated. Optimal nitrogen removal performance (C/N ratio of removal 2.1 kg COD/kg N and overall nitrogen removal rate 0.27 mM h^{-1}) was observed where the specific flow rate (L gX^{-1} h^{-1}) was 10 fold higher through the nitrification column than through the denitrification column. The performance was not improved by the direct cycling of reactor liquor from the denitrification column to the nitrification column (C/N ratio of removal 3.1 kg COD/kg N and overall nitrogen removal rate 0.17 mM h^{-1}). Strategies to decrease the energy cost for recirculating reactor liquor were investigated. Intermittent pumping of reactor liquor through the denitrification column during the nitrogen removal phase decreased the energy input while maintaining good nitrogen removal performance (C/N treatment ratio 2.0 kg COD/kg N and overall nitrogen removal rate 0.21 mM h^{-1} for operation of the denitrification pump 1.7 minutes in every 30 minutes during the nitrogen removal phase). There was, however, no clear relationship between power expenditure and nitrogen removal outcome. Except in terms of pH reduction inhibiting nitrification and ammonia washout from the denitrification column, the reactor performed better under conditions resembling multistage treatment than PND treatment. The storage driven denitrification reactor could be stored for lengthy periods (3 months) at 4°C without long term effect on the nutrient removal performance.

7.1 Process control parameter manipulation for optimisation of PND

To optimise the removal of nitrogen from municipal wastewater, a multistage biofilm reactor was developed in which the nitrification biomass was separated from the denitrification biomass. The individual reactor vessels were then able to be optimised with regard to environmental conditions, for example the supply of oxygen to the nitrifiers and supply of anoxic conditions and a source of COD for the denitrifiers. The operation of the multistage reactor was then in three stages: sequestration of COD under limited oxygen supply by the denitrification biomass, nitrification of the COD free wastewater, and denitrification of the nitrified wastewater using the sequestered COD as electron donor. The resulting reactor was competitive with reactors of both multisludge and single sludge designs (C/N ratio of removal, rate, % N). The key drawback of the multistage system however was that the proton production during ammonia oxidation was not balanced by proton consumption *via* denitrification as it would be in a single sludge reactor. The reduction of pH inhibited nitrification activity.

The PND reactor was developed in order to address the lack of stability in the operational pH of the multistage system. In the PND process, a nitrogen and COD rich wastewater is first supplied to the denitrification biofilm, as in the multistage system and then the ammonia rich wastewater was then passed simultaneously through the denitrification and nitrification biofilms. The PND reactor was competitive with the multistage reactor in terms of performance (< 4.0 kg COD/kg N) and successfully managed the reduction in pH previously observed by the multistage process.

While the PND system performed well, the actual amount of mixing between the two biofilm vessels that is required to achieve complete nitrogen removal was not investigated. The mixing between the two chambers could be achieved in a variety of ways: intermediate batch operation of the process between completely multistage (sequential) and completely PND processes, intermittent operation of the denitrification recycle pump and manipulation of the relative rate of liquor flow within each biofilm chamber. The aim of this chapter was to investigate the manipulation of the method and rate of liquor exchange between the nitrification and denitrification chambers in order to approach an optimised operation. As there is expected to be a cost component related to flow rates used [82], the investigation was extrapolated to suggest a cost minimised optimal performance also.

In addition, the ability of the reactor to be stored during lengthy idle periods at 4°C was investigated.

7.2 Materials and Methods

7.2.1 Laboratory reactor design and operation

All configurations of the PND reactor used in this chapter employed the same acetate uptake phase as the storage driven denitrification, multistage and PND reactors in previous chapters. The reactor design used for the work in this chapter was identical to that described in Chapter 5 (Section 5.2.1) with the exception of when the series *versus* parallel liquor flow was tested. The lengths of the two phases in the trials are detailed in

the results and discussion section where appropriate but, as in previous configurations, the fill and decant phases were rapid (< 3% total cycle time).

In all experiments testing flow rate and direction, the air flow inlet was directed into the headspace of the nitrification reactor at a flow rate of 0.1 L/min controlled with a solenoid switching air flow on for 0.25 minutes in every 6 minutes. The feed supplied to the reactor was the same as that used for the multistage system (Section 4.2.1) unless otherwise specified in the results and discussion. Where changes were necessary, this was only in terms of the C/N ratio of the influent feed.

Manipulation of relative recirculation rate through the nitrification and denitrification columns

The parallel operation (Figure 5.1), where each reactor had a pump that could be independently controlled with respect to flow rate allowed a manipulation of the ratio of the relative recycle speeds in each of the reactors. Variation of relative flow rate resulted in altered k_La during the nitrogen removal phase for the reactor (Table 7.1). The k_La for acetate uptake phase was unchanged.

Serial operation of the PND reactor

To test the effect of direct (serial) transfer of liquor from the denitrification reactor to the nitrification reactor rather than indirect (parallel) transfer of liquor, the PND reactor was modified to allow direct transfer of reactor liquor exiting the denitrification vessel to the nitrification vessel. A pair of solenoids acted as a gate for reactor liquor (Figure 7.1). The acetate uptake phase occurred as normal with liquid circulated in the storage

driven denitrification reactor only. During the nitrogen removal phase, the gate changed and liquor was allowed to transfer directly from the denitrification column to the nitrogen column and back to the recycle vessel (Figure 7.1).

Table 7.1 Experimental conditions for the variation in flow rate ratio and flow direction experiments.

Flow rate through nitrification column		Flow rate through denitrification column		Approximate exchange ratio (N:DN)[23]	Feed C/N ratio (kg COD/kg N)	k_La[24] (h^{-1})
mL min^{-1}	L $gX^{-1}h^{-1}$	mL min^{-1}	L $gX^{-1}h^{-1}$			
50	5.8	500	0.60	10	2.67	0.15
178	21	178	0.21	100 (parallel)	1.89	21.7
178	21	178	0.21	100 (serial)	2.30	13.3
200	23	20	0.024	1000	2.14	16.3

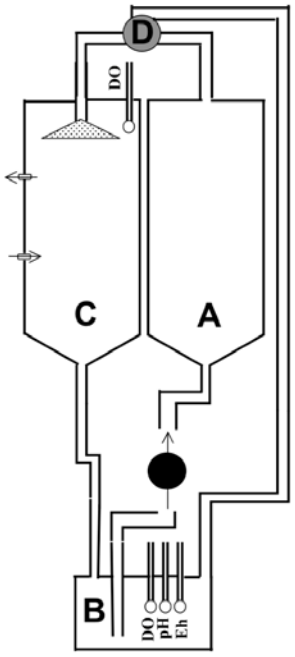

Figure 7.1 The reactor configuration for the serial recycle mode experiment in which the two columns were operated in series rather than in parallel during the nitrogen removal phase. An acetate uptake phase identical to previous PND tests (Section 5.2.1) was used prior to the nitrogen removal phase. During acetate uptake, the reactor liquor flowed only between the denitrification column (A) and the recycle vessel (B) as directed by the solenoid gate (D). After completion of the acetate uptake phase, the solenoid gate switched to allow reactor liquor to flow from the recycle vessel (B), through the denitrification column (A) to the nitrification column (C) before returning to the recycle vessel (B). The recycle pump is indicated by a solid circle with arrow for direction of flow.

[23] See Section 7.2.3 for details of calculation.

[24] k_La values for PND phase only. During acetate uptake, k_La was 1.1 h^{-1} or less.

Intermediate operation of the reactor between multistage and PND

To test the effect of intermediate operation between multistage and PND systems, the reactor control program was modified to allow exposure of the liquor to only one reactor at a time. That is, once the nitrogen removal phase had started, the whole of the reactor liquor was transferred to the nitrification column for a defined time (Section 7.3.3) and then transferred back to the denitrification column for a defined time. The process was repeated until ammonia removal was complete and nitrogen removal was complete or near to complete. During the nitrification phases, the denitrification column was degassed with nitrogen and then sealed to minimise oxidation of PHB.

Intermittent exchange of reactor liquor

With intermittent exchange of reactor liquor, the nitrogen removal phase occurred with discontinuous recirculation through the denitrification column while the nitrification recirculation was maintained through the nitrification column. The actual length of time during which the denitrification pump is operated is given in the relevant results section.

7.2.2 Analysis

All nitrogen and phosphorus compounds were measured spectrophotemetrically (Section 2.2.4). Acetate was measured by gas chromatography (Section 2.2.4).

7.2.3 Calculations

Measurement of k_La, calculation of OUR and hence oxygen consumed, C/N ratio of removal and overall rates of nitrogen and ammonia removal were performed as described previously (Section 5.2.3).

Effective liquor exchange rate estimation

Due to the very different design of the two column reactors involved in the PND reactor (trickling filter *vs* submerged approximate plug flow), it was difficult to describe a consistent parameter for the flow rate of liquor in each. The biomass contained in each biofilm was estimated (Appendix F) and the specific liquor application rate (L gX^{-1} h^{-1}) was calculated for each reactor (Table 7.1). The ratio of flow rates used (nitrification:denitrification) was then used to estimate the liquor exchange ratio (Table 7.1).

7.3 Results and Discussion

The flow of liquor within each chamber of the PND reactor is designed to achieve mixing and oxygen delivery in the aerobic trickling filter nitrification reactor and mixing in the denitrification reactor. Greater mixing achieved through an increase in flow rate is expected to improve substrate diffusion into each biofilm and hence improve reaction rate. Results of this type have been observed previously (denitrification: Section 3.3.4 and nitrification Section 4.3.1).

The purpose of the study in this chapter was not to further investigate the individual effect of flow rate on reaction rate, but instead to focus on the effect on nitrogen removal performance by the amount of exchange between the two reactors. A change in the effective exchange of liquor between the two chambers is likely to affect the delivery of substrate to each reactor. Consider, for example, the production of nitrite or nitrate in the nitrification reactor. Where the denitrification recirculation pump is not operated, the denitrification chamber will not receive nitrite for denitrification and hence COD may be wasted due to the stoichiometric production of nitrate from ammonia. The exchange amount between the two reactors may be achieved in a number of ways and each will be investigated in turn:

- variation of the relative rate of liquor flow within each biofilm chamber;
- intermediate batch operation of the process between completely multistage and completely PND processes; and
- intermittent operation of the denitrification recycle pump.

7.3.1 Effect of relative recycle speed on PND performance

One method to alter the exchange of liquor between the nitrification and denitrification reactors, was to alter the relative flow rate (recycle speed) in each reactor. The design of the reactor used in developing the PND is such that each chamber independently sources and drains through a common recycle vessel (Figure 5.1). In each test, different absolute flow rates were used (Table 7.2) in each chamber which supports the previously observed phenomenon in which the flow rate affected the reaction rate in

each reactor (Sections 3.3.4 and 4.3.1). This must of course be acknowledged as a potential confounder of the results on the effect of relative flow rates used.

Table 7.2 Effect of exchange ratio on nitrogen removal rates of the PND reactor.

Exchange ratio (N:DN)[25]	**C/N ratio of removal (kg COD/kg N)**	**Overall rate of N removal (mM h^{-1})**	**Rate of nitrification during PND phase (mM h^{-1})**	**Rate of denitrification during PND phase (mM h^{-1})**	**Peak accumulation of intermediates (%)[26]**	
					Nitrate	Nitrite
10	3.3	0.18	0.24	0.22	6.4%	1.3%
100	2.1	0.27	0.45	0.38	8.8%	8.7%
1000	4.0	0.15	0.36	0.24	34%	3.0%

The change in relative flow rates in each reactor did not have a clear effect on the overall rate of nitrogen removal (Table 7.3 and Figure 7.2). However, it is clear that the overall kinetics of nitrogen removal changes with change in relative flow rate (Table 7.3). The removal of nitrogen in the PND reactor was previously observed to be first order with respect to ammonia due to the limitation of substrate diffusion affecting the underlying enzyme kinetics (Section 5.3.2). The substrate diffusion limitation of nitrogen removal was observed again under the relative flow rate conditions except where the exchange ratio (Section 7.2.3) was lowest (Table 7.3). The first order rate observed, with respect to total nitrogen, indicates that under conditions of exchange ratio at 100 or 1000, the reactor was limited by the rate of denitrification allowed due to either the lower absolute flow rate or the lower exchange ratio (Table 7.2). The considerably higher accumulation of nitrate and nitrite at an exchange ratio of 1000 than at 100 does not allow clear differentiation of which explanation dominates. However, a simple model consisting of three completely mixed flow reactors (Appendix H) in which the relative flow rates were varied indicates that the exchange ratio could indeed

[25] See Table 7.1 for individual flow rates and calculation of exchange ratio.

[26] Peak accumulation of intermediates expressed as percent of influent ammonia.

be responsible for the reduced overall nitrogen removal rate observed at an exchange ratio of 1000 (Table 7.3).

Table 7.3 Effect of exchange ratio on the nitrogen removal kinetics of the PND reactor.

Exchange ratio (N:DN)[27]	**C/N ratio of removal (kg COD/kg N)**	**Kinetics of ammonia oxidation**	
		Apparent reaction order (R^2)	Rate constant
10	3.25	Zero order (R^2 = 0.9756)	0.22 mM h^{-1}
100	2.06	First order (R^2 = 0.9764)	0.54 h^{-1}
1000	4.03	First order (R^2 = 0.9756)	0.34 h^{-1}

When the PND reactor was operated with an exchange ratio of 10 (Tables 7.2 and 7.3), the reactor appeared to be limited by nitrification activity. The accumulation of nitrite and nitrate was lower under this condition indicating that the transfer of nitrite and nitrate produced *via* nitrification to the denitrification biofilm was better than at higher exchange ratios (Table 7.3).

Due to the potential confounding of the effect of exchange ratio by the absolute flow rates used in each reactor (Table 7.2), it is difficult to definitively suggest an optimal exchange ratio for PND operation. It would appear however that an exchange ratio of 100 may be optimal as it has the highest rate of nitrogen removal, relatively low accumulation of NO_x and the lowest C/N ratio of removal achieved (Table 7.3). The relative amount of biomass in each reactor (0.5 g of nitrifiers and 50 g of denitrifiers; ie approximately 100 times as much denitrification biomass as nitrification biomass) reflects that found in mixed sludge reactors. The flow rate (L h^{-1}) in each chamber is equal at an exchange ratio of 100 (Table 7.2) and this suggests that the ratio of

[27] See Table 7.1 for individual flow rates and calculation of exchange ratio.

nitrification biomass to denitrification biomass in the PND reactor is optimal. Further study would be required to confirm this result.

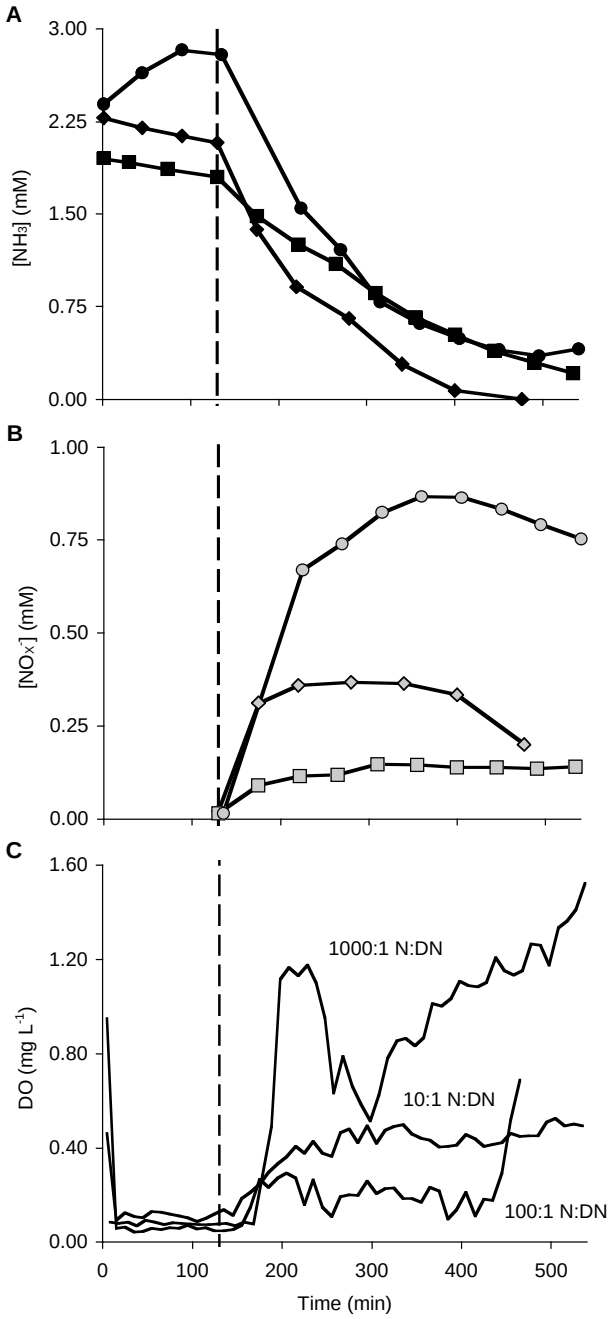

Figure 7.2 Effect of relative specific flow rate in each column during nitrogen removal. Three relative specific flow rates were tested (N column : DN column; 10, 100, 1000; See Table 7.1 for details). In Figures A and B, diamonds represent 100:1 relative specific flow rate N:DN, circles represent 1000:1 relative specific flow rate N:DN and squares represent 10:1 relative specific flow rate N:DN. Vertical dashed line indicates phase change from acetate uptake phase (130 minutes) to PND phase (>350 minutes).

7.3.2 Effect of recycle mode on PND performance

From the results above (Section 7.3.1), it appears that the relative amount of biomass contained in each biofilm of the PND reactor is optimal and leads to the optimal relative flow rates (L h^{-1}) to be equal. The use of a common recycle vessel from which each biofilm chamber sources and drains (Figure 5.1) under optimal conditions (equal flow rate, 100 times as much nitrification biomass as denitrification biomass) then leads to the consequence that any NO_x produced in the nitrification reactor has only a 50% chance of migrating to the denitrification reactor as opposed to returning to the nitrification column. Under this parallel operating condition, any nitrite that returns to the nitrification column would waste oxygen in the nitrification column (due to further oxidation to nitrate) and COD in the denitrification column (due to denitrification substrate becoming nitrate instead of nitrite) and thus any method to minimise this effect was expected to improve the overall performance of the reactor.

An alternative reactor design was tested (Section 7.2.1; Figure 7.1) in which the liquor exiting the denitrification column was passed serially through the nitrification column before proceeding to the recycle vessel. The overall rate of nitrogen removal was higher under parallel operation than in serial operation (0.27 *vs* 0.17 mM h^{-1}; Table 7.4) and a reduced carbon requirement for nitrogen removal (2.1 *vs* 3.1 kg COD/kg N; Table 7.4) was observed. While the same level of nitrate and nitrite were accumulated under each flow regime (Table 7.4), the time at which the maximum concentration was reached differed (Figure 7.3). In addition, the apparent zero order with respect to ammonia reaction of ammonia oxidation under serial operation indicated that the nitrification biofilm was probably substrate saturated (Table 7.4).

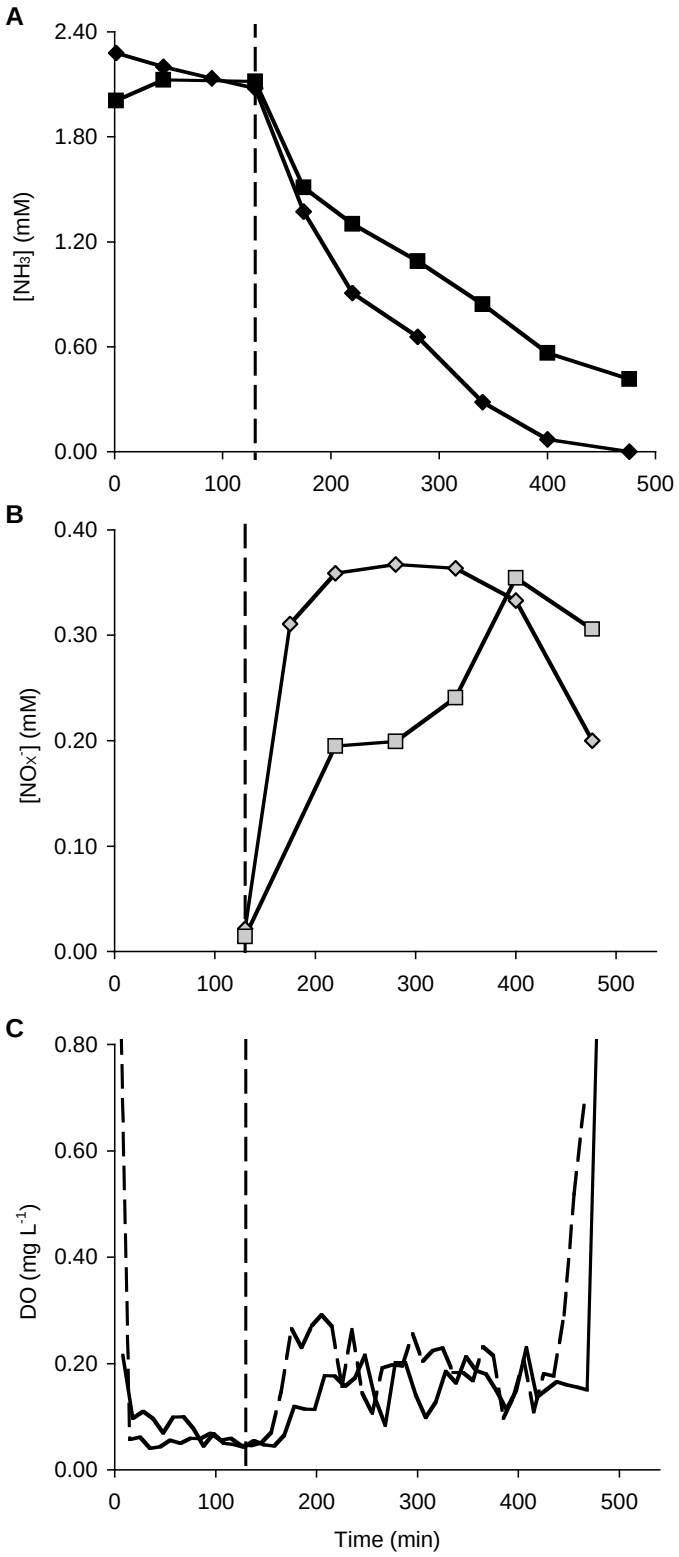

Figure 7.3 Effect of direct liquor transfer between each column during nitrogen removal. In both trials, the exchange ratio of was 100:1 N:DN (see Table 7.1 for details). In Figures A and B, diamonds represent parallel flow (Section 7.2.1) between the N and DN columns, circles represent serial flow (Section 7.2.1) between N and DN columns. In Figure C, dashed line represents parallel flow and unbroken line represents serial flow. Vertical dashed line indicates phase change from acetate uptake phase (130 minutes) to PND phase (350 minutes).

Table 7.4 Effect of flow direction (parallel or serial) on the nitrogen removal activity of the PND reactor.

Flow regime during PND phase	**C/N ratio of removal (kg COD/kg N)**	**Overall rate of N removal (mM h^{-1})**	**Kinetics of ammonia oxidation**		**Peak accumulation of intermediates (%)**	
			Apparent reaction order (R^2)	Rate constant	Nitrate	Nitrite
Parallel[28]	2.06	0.27	First order (R^2 = 0.9764)	0.54 h^{-1}	8.8%	8.7%
Serial	3.12	0.17	Zero order (R^2 = 0.9883)	0.22 mM h^{-1}	7.9%	9.8%

A simple model was constructed to test the effect of a serial *vs* parallel flow regime (Appendix H) and was found to give superior nitrogen removal under serial operation of the reactor as would be expected (rate under serial ~1.4 times faster than parallel operation; Figure 7.4). The discrepancy between the expected result, supported by the theoretical model, and the experimental data may be the result of increased oxygen delivery to the denitrification reactor inhibiting denitrification. The oxygen mass transfer coefficient of the parallel process was higher than the serial process (Table 7.1). The oxygen present in the recycle vessel under serial flow conditions however, has a considerably higher chance of entering the denitrification reactor than under serial operation analogous to the behaviour of NO_x under parallel *vs* serial flow.

While the performance appeared to be superior under parallel operation over serial operation, it should be noted that under either flow regime, the performance of the PND system was still superior to many alternative designs (Section 5.3.3). The use of the serial flow regime may reduce the cost of operating a larger scale PND reactor by up to half (one pump instead of two) and still achieve an efficient removal of nitrogen.

[28] Parallel operation data is reproduced from Tables 7.2 and 7.3 experiment with exchange ratio of 100 (Section 7.2.3) during PND phase.

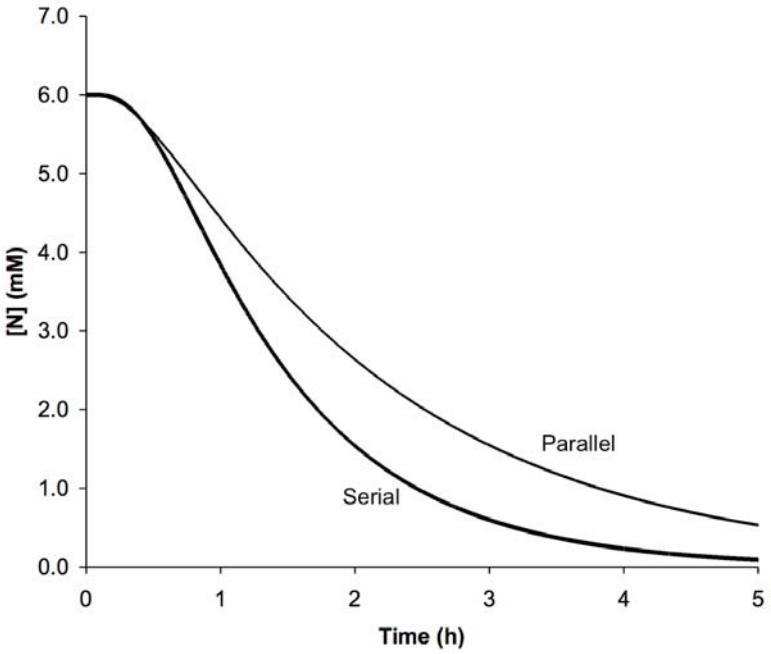

Figure 7.4 Modelled[29] effect of direct liquor transfer between the nitrification and denitrification chambers. Total nitrogen concentration in the recycle vessel shown under direct transfer (serial) operation (bold line) and under indirect (parallel) operation.

7.3.3 Intermediate operation between multistage and PND

The PND reactor was a clear improvement over the completely separate batch operation under multistage conditions (Section 7.1). Just how much balancing of proton production *via* denitrification is required was not clear. The intermediate operation of the reactor with varying short batch phases of nitrification and denitrification was tested in order to establish if complete PND operation was required to achieve stability in the observed process pH (Section 7.2.1).

Net removal of nitrogen generally only occurred during the denitrification phases in all trials (Figure 7.5), as expected. Considerably higher than normal PND buildup of nitrate

[29] Details of model and parameters given in Appendix H.

was observed in all trials, up to 50% of the total nitrogen entering nitrogen removal phases. This was expected as the cycling through a single reactor limits treatment to only that allowed by that reactor and hence, as in the 10:1 ratio flow rate experiment above, nitrite produced has more chance of being further oxidised to nitrate rather than passed to the denitrification reactor for reduction.

The pH was shown to change in the different phases however the large changes in pH due to the oxidation of ammonia were not seen and the pH remained high enough to limit inhibition of nitrifiers (Figure 7.5; pH >7).

As expected in all three tests (batch time 30, 60 or 120 minutes; Section 7.2.1), ammonia was removed during the nitrification phases and relatively stable during the denitrification phases (Figure 7.5). In some denitrification phases, particularly in the 60 minute cycle experiment (Figure 7.5*B*), ammonia concentration increased during denitrification which was due to the observed washout from the acetate uptake phase (Appendix E).

This set of trials increased the foot print of the system due to the need for holding tanks between each phase, but highlighted a possible intermediary operation that could still achieve pH control compared with full PND operation (Chapter 5). The performance of the system was greatest in terms of C/N ratio removal achieved in the 120 minute cross over cycle which in fact allowed for complete nitrification of all influent ammonia and then subsequent denitrification in the first denitrification phase. Additional phases subsequent to this were only required to remove the ammonia washed out of the denitrification reactor in successive cycles. The C/N ratio of removal of the 120 minute crossover test was 2.2 kg COD/kg N while for the 30 and 60 minute crossover tests it

was 3.5 and 2.6 kg COD/kg N respectively (Table 7.5). This indicated that the low C/N ratio of removal observed in the PND and multistage systems may be due to a combination of ammonia assimilation[30] and low growth requirement for COD or luxury storage rather than a short circuit between nitrification and denitrification *via* nitrite (Section 5.3.5).

As long as the reactor liquor pH was stabilised, the removal of nitrogen could be improved by operating closer to the completely separated multistage system. Based on the previously discussed nitrogen removal assessment criteria (Section 4.3.5), the optimal cycle time tested appeared to be 120 minute separated phases for nitrification and denitrification.

Table 7.5 Effect of intermediate operation of PND reactor on nitrogen removal.

Phase time (minutes)[31]	**Feed C/N ratio (kg COD/kg N-NH_3)**	**C/N ratio of removal**		**Overall rate of nitrogen removal (mM h^{-1})**	
		kg COD/kg N-NH_3	kg COD/kg N	Ammonia	Nitrogen
30	2.8	2.8	3.5	0.44	0.30
60	2.1	2.1	2.6	0.49	0.32
120	2.0	2.0	2.2	0.52	0.30

[30] While the amount of ammonia assimilated would depend on the C/N ratio of the feed (Appendix D), it can be roughly calculated based on the calculated amount of COD assimilation. Where 9% of influent COD (feed concentration at 2.4 mM acetate; Section 2.2.1) is assimilated, this corresponds to 0.21 mM acetate or 0.43 CmM. The general formula for biomass ($C_5H_7NO_2$ [122]) then leads to 0.09 mM ammonia assimilation. In a standard feed (feed concentration at 2.9 mM ammonia; Section 2.2.1) this represents only 3% of influent ammonia.

[31] Phase time refers to the length of each alternate nitrification and denitrification phase after acetate uptake phase completion.

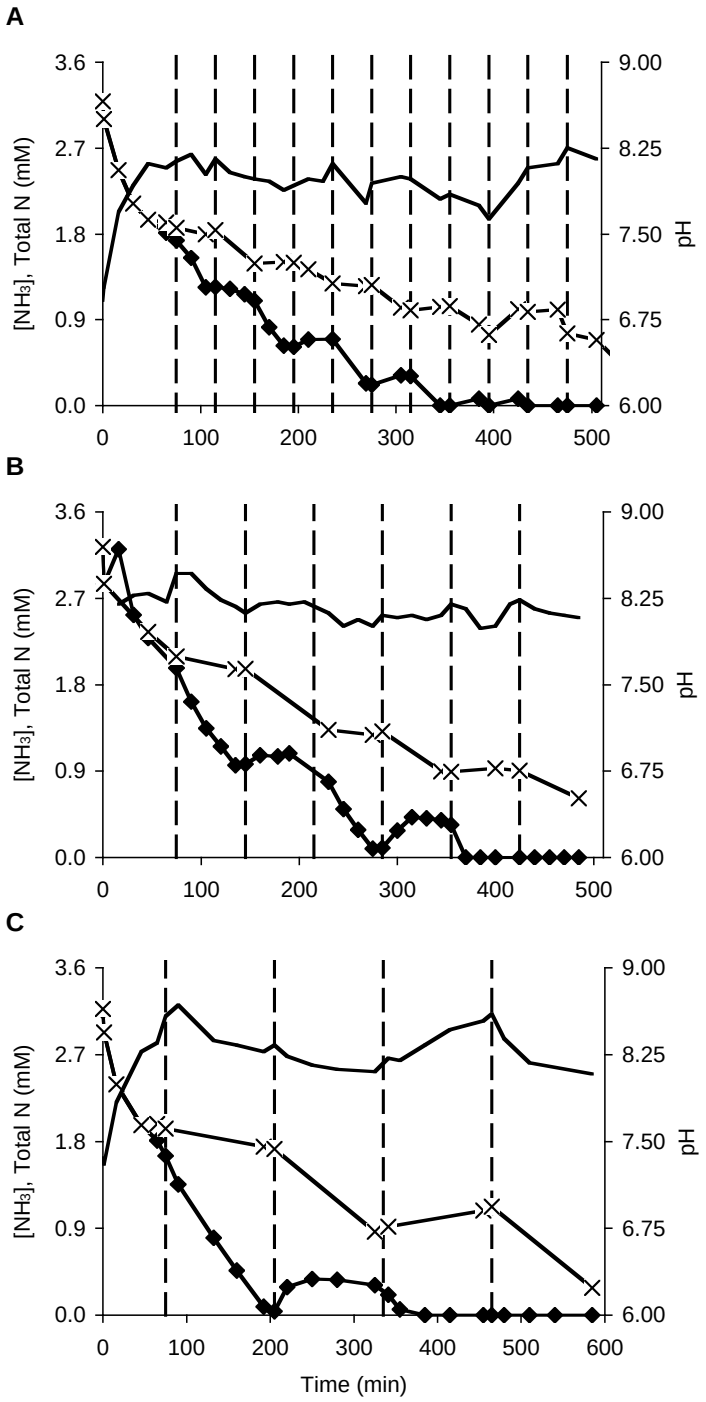

Figure 7.5 Effect of intermediate operation, between PND and multistage, on nitrogen removal of the PND reactor. After an initial identical acetate uptake phase, the reactor liquor was transferred completely to the nitrification column for 30 minutes (A), 60 minutes (B) or 120 minutes (C) and then back to the denitrification column for the same length of time. The sequence was repeated until ammonia removal was complete. The denitrification reactor was flushed and stored under nitrogen gas while idle. Ammonia (◆) and total nitrogen (×). Vertical dashed line indicates phase change.

7.3.4 Effect of intermittent operation of the denitrification recirculation pump

The results of the intermediate operation of the reactor between fully multistage and fully PND above (Section 7.3.3) suggest that the optimal removal of nitrogen can be achieved by the limitation of the denitrification phase until an appreciable concentration of substrate for denitrification had been achieved. The limitation of the denitrification phase can be achieved by the intermittent operation of the denitrification recirculation pump. A trial was conducted to test the intermittent operation of the denitrification recirculation pump in which it was alternately switched off for 50% of reaction time. As the performance of the reactor was comparable to previous trials (Figure 7.6 and Table 7.6), an additional trial was conducted to minimise pumping in the denitrification column further. The denitrification pump was operated for only 5% of reaction time. As a result, at the start of each phase change, the pH and ammonia and total nitrogen concentrations increased due to the fresh feed being added (Figure 7.7). Ammonia removed was approximately stoichiometrically converted to nitrate (Figure 7.7), however the total amount of nitrogen in the system was fairly stable over time (Figure 7.7). It was clear that although nitrification was occurring, denitrification was not substantially converting the accumulated nitrate into nitrogen gas. The stagnation of reactor liquor in the denitrification column during idle periods may have allowed diffusion limitation of nitrate and nitrite to the biomass to become significant.

The shorter pumping time compared to the previous trial (Figure 7.6) resulted in superior nitrogen removal performance in terms of C/N ratio of removal (Table 7.6). However, it did appear that while the overall rate of nitrogen removal was relatively unchanged between these two trials (Table 7.6) a limitation of nitrogen removal seemed to be reached in the trial with lower denitrification pumping (Figure 7.7 *vs* Figure 7.6).

Although it is difficult to compare the batch PND reactor directly with alternative designs from literature, the intermediate use of pumping here or intermittent reactor use (Section 7.3.3) is analogous to the use of intermittent aeration to encourage alternating conditions encouraging nitrification and denitrification. Yoo *et al* [98] observed that short aeration periods encouraged the activity of nitritifying organisms and encouraged nitrogen removal *via* nitrite due to aeration length kept short compared with the lag time of nitratifying organisms. Similarly, the long periods of non aeration employed by Mota *et al* [123] optimised nitrogen removal *via* nitrite.

Habermeyer and Sanchez [124], studying simultaneous removal of COD and nitrogen by intermittent aeration achieved optimal nitrogen removal (100% of COD and ammonia with a feed of 20 kg COD/kg N-NH_3) when the aeration and non aeration phases (120 minutes each) were very long allowing full conversion of influent ammonia to nitrate prior to denitrification. As in this study, those operating with conditions resembling the completely separated multistage system appear to perform best.

Intermittent pumping of reactor liquor through the denitrification column did seem to be a successful strategy to reduce energy costs while maintaining an adequate nitrogen removal performance. Those trials that operationally resemble the multistage system appear to have better nitrogen removal performance but still require management of pH and ammonia washout.

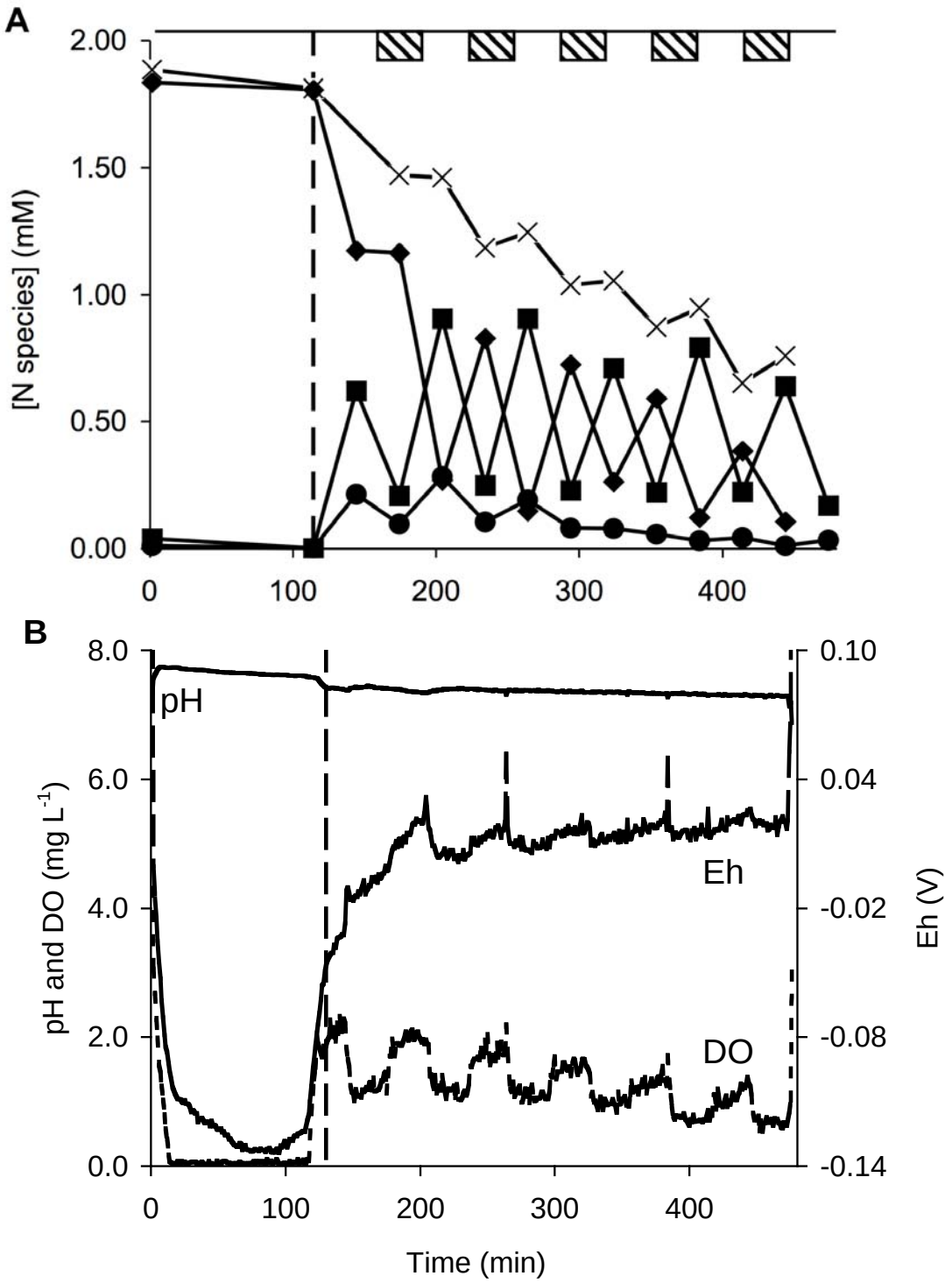

Figure 7.6 Effect of intermittent recirculation of liquor during the nitrogen removal phase. The denitrification recirculation pump was switched off at the start of the nitrogen removal phase for 30 minutes and then on for 30 minutes. The sequence was repeated until the end of the cycle. Synthetic wastewater (Feed Ammonia 1.8 mM and Acetate 1.5 mM; 3.6 kg COD/kg N-NH_3) as substrate: Ammonia (◆), nitrate (■), nitrite (●) and total nitrogen (×). Vertical dashed line indicates phase change, times of activation of denitrification pump shown by bars at top of A.

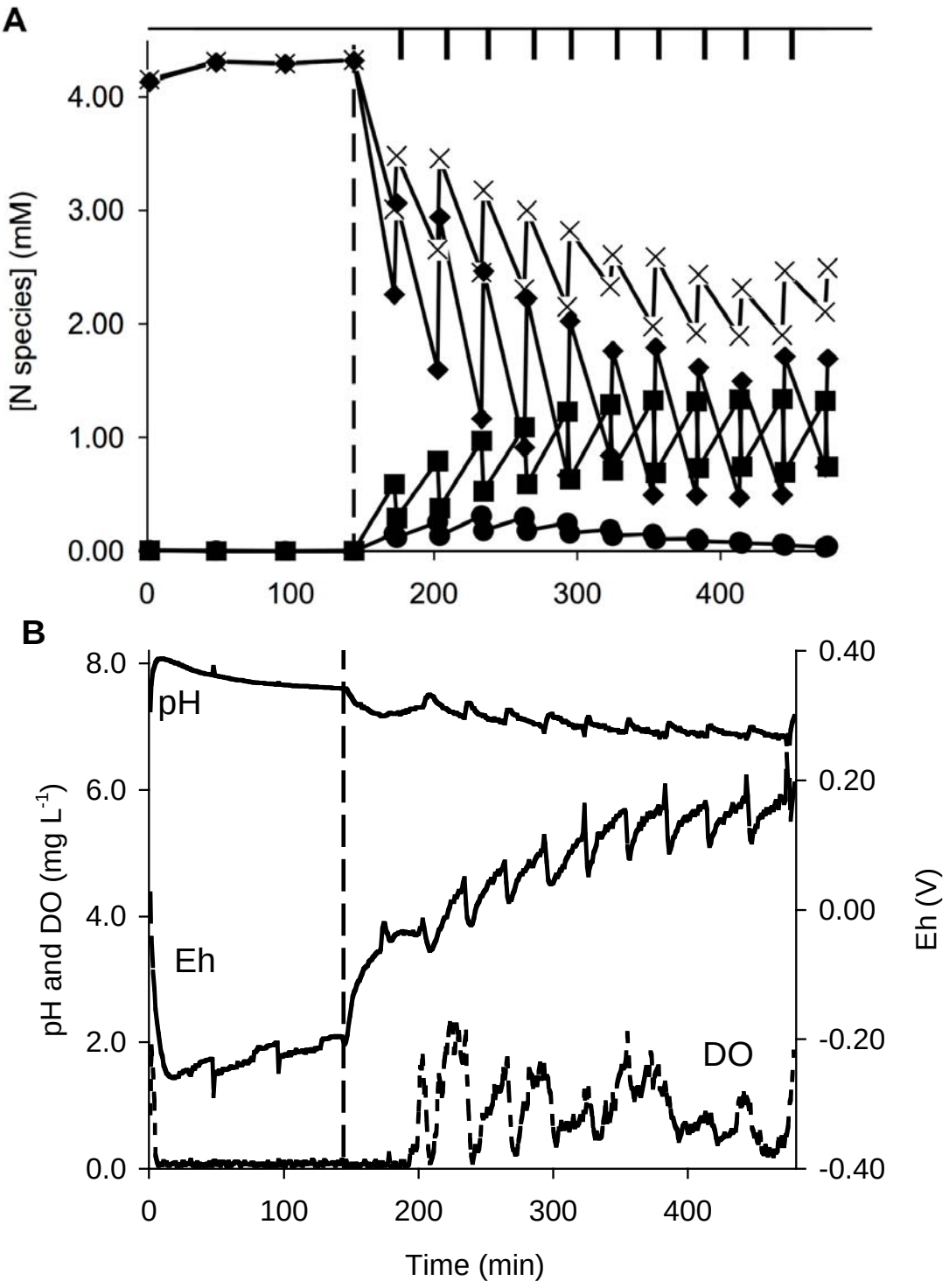

Figure 7.7 Effect of intermittent recirculation of liquor during the nitrogen removal phase. The denitrification recirculation pump was switched off at the start of the nitrogen removal phase and operated for 1.7 minutes in every 30 minutes until the end of the cycle. Synthetic wastewater (Feed Ammonia 4.1 mM and Acetate 1.7 mM; 1.8 kg COD/kg N-NH_3) as substrate: Ammonia (◆), nitrate (■), nitrite (●) and total nitrogen (×). Vertical dashed line indicates phase change, times of activation of denitrification pump shown by bars at top of A.

Table 7.6 Effect of intermittent operation of the denitrification recirculation pump on nitrogen removal by the PND reactor.

Denitrification pump setting[32]	**Feed C/N ratio (kg COD/kg N-NH_3)**	**C/N ratio of removal**		**Overall rate of nitrogen removal (mM h^{-1})**	
		kg COD/kg N-NH_3	kg COD/kg N	Ammonia	Nitrogen
5%	1.8	1.4	2.0	0.31	0.21
50%	3.6	3.9	4.4	0.20	0.18

7.3.5 Potential optimal power consumption for nitrogen removal *via* PND

The flow rates employed in the lab scale reactor in this thesis to achieve mixing of the reactor liquor either within an individual biofilm chamber or between the biofilm chambers fall within a reasonable pumping rate employed in large scale systems (*eg* 160 L s^{-1} at the Woodman Point WWTP). The cost of pumping is expected to be higher where higher flow rates are used [82]. While this relationship (cost of operation *vs* flow rate) is dependent on the specific load on the pump motor and may not be linear, it is worth considering potential cost savings by reduced pumping required. There is no clear relationship between the relative power input and the performance of the reactor in terms of C/N ratio of removal achieved or the overall rate of nitrogen removal (Figure 7.8). The percentage removal of nitrogen does appear to have been limited at lower power inputs (Figure 7.8*B*).

[32] Denitrification pump was only operated for the designated time while the nitrification pump was operated continuously during the nitrogen removal phase.

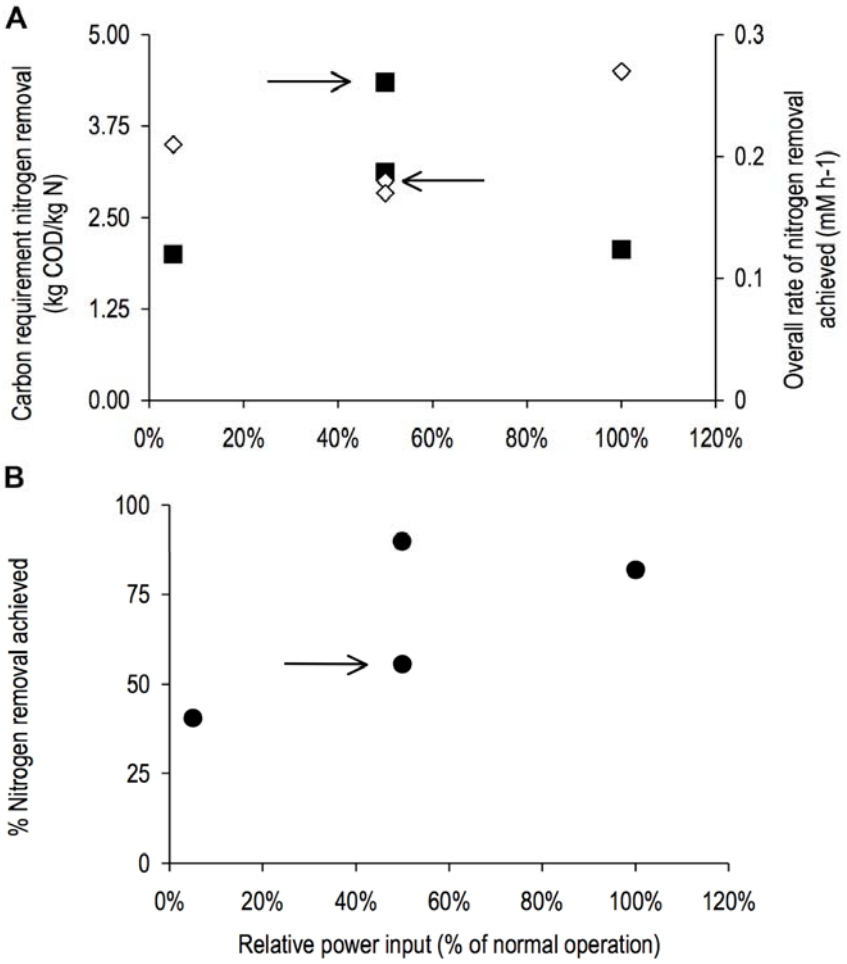

Figure 7.8 Effect of relative power input[33] on the nitrogen removal performance of the PND reactor. Arrows indicate data for 50% intermittent operation of the denitrification recirculation pump (Section 7.3.4).

7.3.6 Effect of refrigeration on the PND reactor

Over the course of this study, occasional storage of the reactors was required due to lengthy idle periods. The effect that storage had on the individual biofilms needed to be understood so that the robustness, or otherwise, of the reactor could be established. Two storage time periods were tested, the first for one month and the second for 3 months.

[33] Relative power input expressed with respect to the amount of liquid flow per unit time. For example, for serial operation, there is 50% power consumed relative to 'normal' parallel operation.

The columns were degassed with nitrogen, sealed and stored at 4°C. The outcome of each time period was similar and as such, only the longer period results will be discussed.

The nitrification reactor had no activity after either storage period indicating that the ammonia oxidising bacteria in that biofilm had an absolute requirement for oxygen, as expected. As a result, after each storage period, the nitrification biofilm had to be re-established as described previously (Section 4.2.2). Due to the lack of sufficient activity of the nitrification column, the storage driven denitrification column had to be operated separately (Section 2.2.2) until sufficient nitrification activity was re-established for reconnection of the PND reactor.

The initial cycle performed after 3 months storage by the storage driven denitrification reactor was different to those routinely observed (*eg* Figure 5.4). A small release of acetate and ammonia was observed from the biofilm during the acetate uptake phase and ammonia continued to be released during the denitrification phase (Figure 7.9). During the storage period, it was likely that with no exogenous food supply, slow endogenous metabolism dominated. As a result, the release of cellular components from death or metabolism could have resulted in the release of VFA material. If this was the case, there may have been no requirement for the cells to uptake externally available acetate to meet their COD requirements. Acetate uptake recovered quickly after the initial cycle (Table 7.7 and Figure 7.10).

Denitrification activity did not appear to be diminished even in the first cycle after storage (Figure 7.9). This supports the hypothesis above that decay of the biofilm during storage supplied abundant exogenous COD for nitrate reduction. The rate of

denitrification during the first cycle was higher than subsequent cycles (Table 7.7) where the cells presumably relied on an internally sequestered carbon source.

The increase in carbon requirement for nitrogen removal from the 7th to the 22nd cycle may have been the result of an increased feed ratio supplied (Table 7.7). It has frequently been observed in this study, that a reduction in supply of COD to the storage driven denitrification biofilm improved the observed C/N ratio of removal (Section 5.3.2). In general, it appeared that the denitrification column recovered quickly from the period of storage (Table 7.7). Refrigeration was an appropriate method of storage during lengthy idle periods.

The loss of ammonia from the bulk liquor during the acetate uptake phase was diminished after storage. The biofilm texture was considerably more granular after storage than prior to storage and indicated that the water content was lower. Lowering the proportion of water in the biofilm matrix would lower the void volume and therefore reduce the capability of adsorbing solute particles from the bulk liquor (Appendix E). Typical ammonia loss during acetate uptake was restored after 1 – 2 months of operation (Figure 5.4).

The storage driven denitrification biofilm could tolerate storage at 4°C for up to four months without long term detriment to reactor activity. However, if full nitrogen removal is required, alternative storage needs to be examined for the nitrification biofilm. As long as a nitrate rich feed source is available to the denitrification reactor then it can be used until the nitrification reactor is performing appropriately.

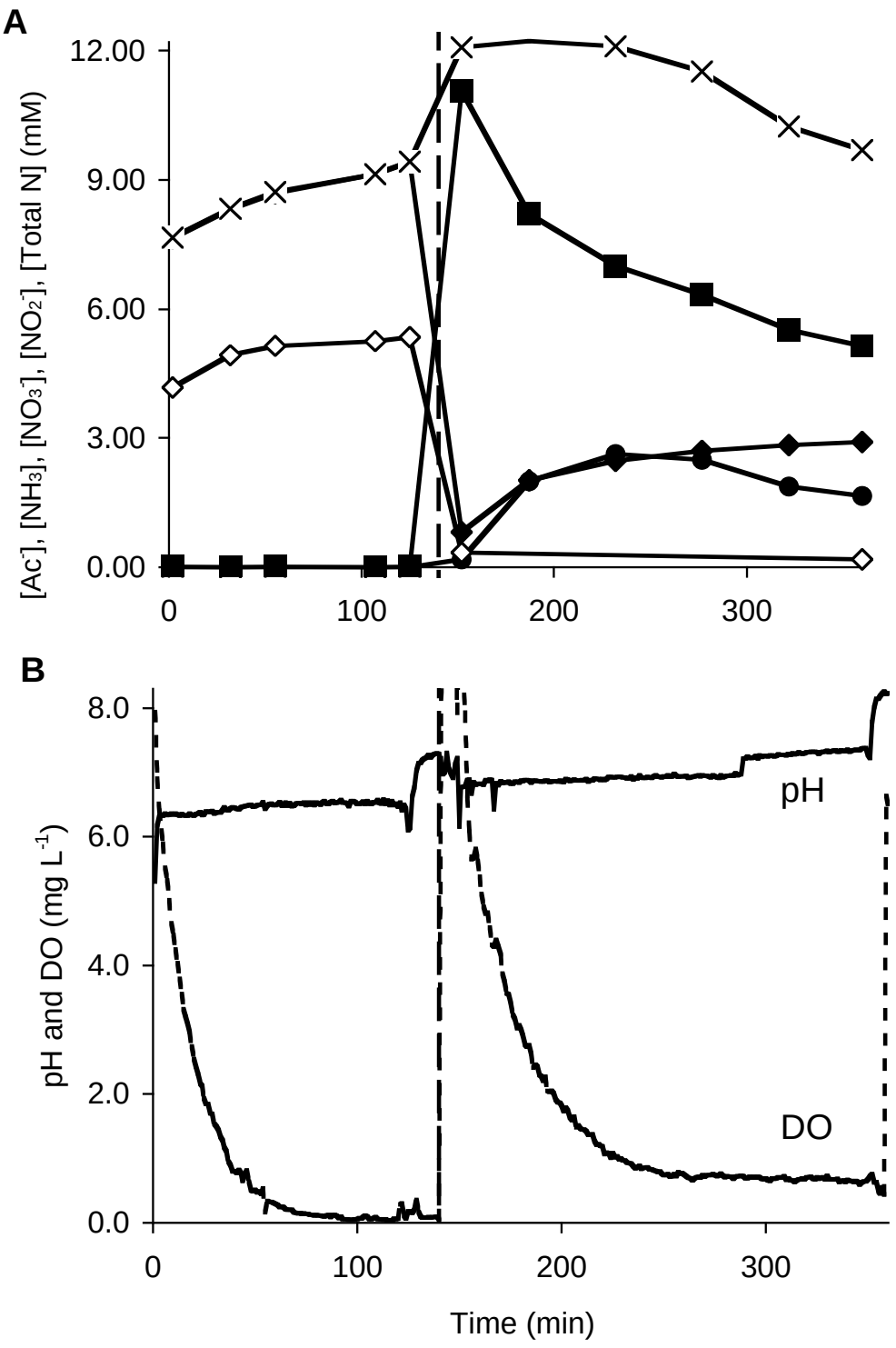

Figure 7.9 Effect of refrigerated (4°C) storage (3 months) on the storage driven denitrification biofilm. The first cycle is shown under storage driven denitrification operating conditions (Section 2.2.2). Synthetic wastewater (Feed during acetate uptake phase acetate 4.2 mM and during denitrification phase nitrate 11 mM; 1.7 kg COD/kg N-NO_3^-) as substrate: Acetate (◇), ammonia (◆), nitrate (■), nitrite (●) and total nitrogen (×). Vertical dashed line indicates phase change.

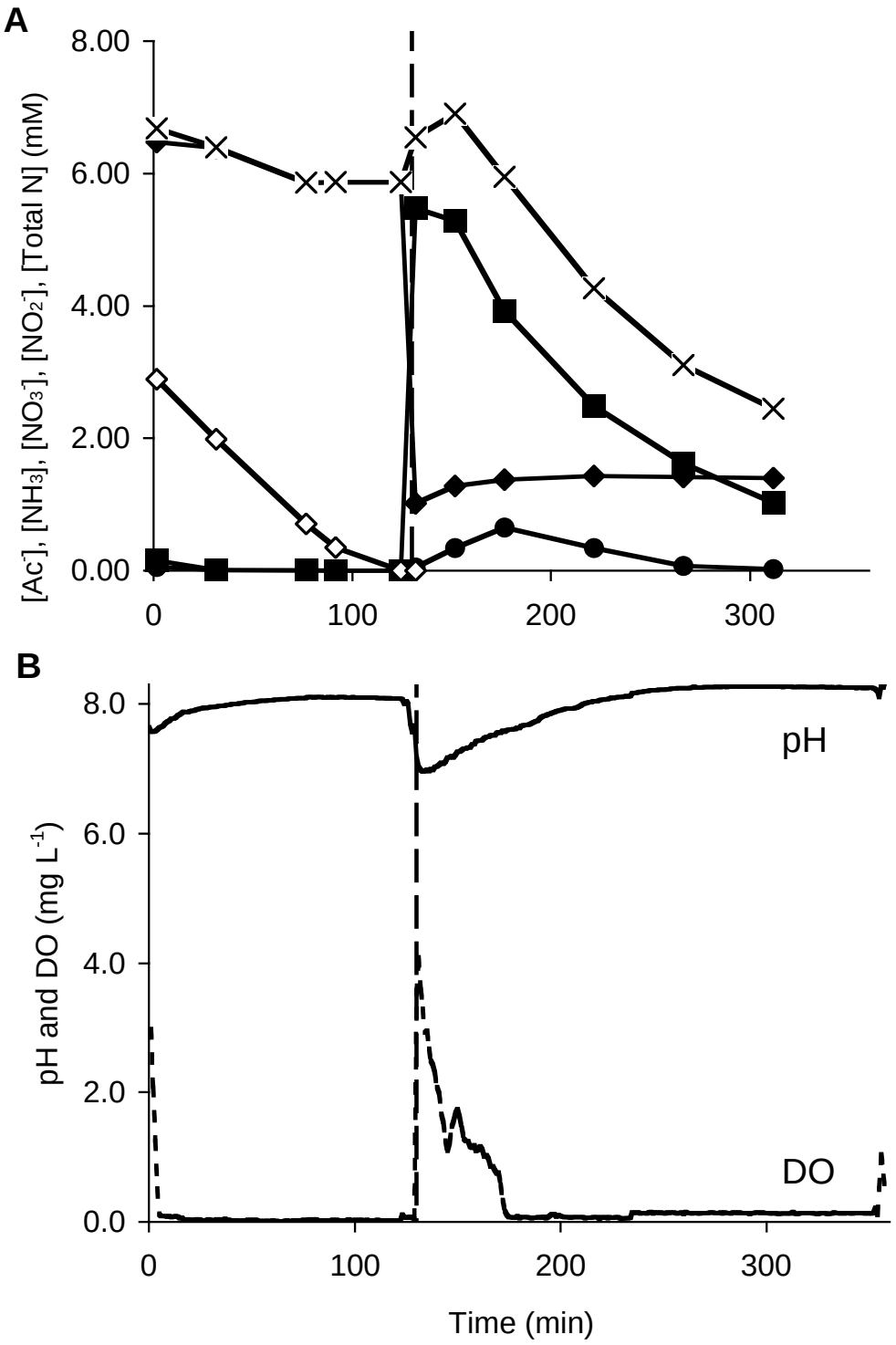

Figure 7.10 Effect of refrigerated (4°C) storage (3 months) on the storage driven denitrification biofilm. The ninth cycle is shown under storage driven denitrification operating conditions (Section 2.2.2). Synthetic wastewater (Feed during acetate uptake phase acetate 6.7 mM and during denitrification phase nitrate 5.5 mM; 2.4 kg COD/kg N-NO_3^-) as substrate: Acetate (◇), ammonia (◆), nitrate (■), nitrite (●) and total nitrogen (×). Vertical dashed line indicates phase change.

Table 7.7 Recovery of the storage driven denitrification reactor after storage for 3 months at 4°C.

Cycle	Feed C/N ratio (kg COD/kg N-NO_3^-)[34]	C/N ratio of removal (kg COD/kg N NO_3^-)	Overall rate of removal (mM h^{-1})	
			Acetate	Nitrate
1	1.7	-0.9	-0.57[35]	1.71
7	2.4	2.6	1.41	1.34
22	3.2	3.1	1.53	1.52

7.3.8 Long-term performance and stability of the reactor systems in this study

The storage driven denitrification reactor was operated for more than two years before combining it with the nitrification biofilm for PND operation. The PND reactor was operated for a further 4 months. During this time, the operation of the reactor was interrupted a number of times and stored (Section 7.3.7). Over the entire length of the study, the activity of each reactor (SDDN and PND) was maintained (Figure 7.11).

The feed used was consistent throughout the study except where higher shock loads were tested and the amount of nitrogen remaining in the effluent was also consistent. Where higher shock loads were used, it can also be seen that the effluent concentration increased accordingly. The amount of ammonia remaining in the effluent was high throughout the study and was improved by washing of the reactor as well as conversion to PND operation.

[34] Feed C/N ratio is based on acetate supply during acetate uptake phase and nitrate supply during denitrification phase

[35] A negative rate shows an increase in concentration, ie a negative rate of loss from the bulk liquor.

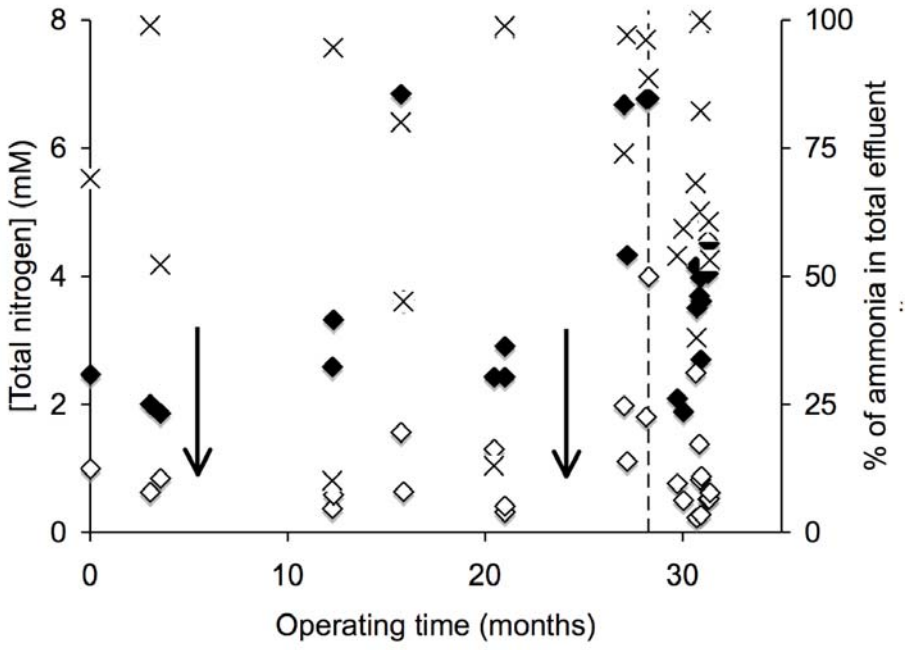

Figure 7.11 Long-term behaviour of the SDDN and PND reactors. Dotted line at 28 months indicates conversion to PND system. Arrows indicate storage at 4°C (Section 7.3.7) for 1 month and 3 months respectively. Influent total nitrogen (◆), effluent total nitrogen (◇) and percentage of ammonia in effluent nitrogen (×).

7.4 Conclusions

The PND system can be manipulated to reduce pumping requirements by altering the method and rate at which the exchange of reactor liquor between the two biofilms occurs. Optimal performance observed by altering the pumping requirements was achieved at maximum relative pumping input, as would be expected. Where the intermediate operation between full multistage and full PND approaches was used, those most closely resembling the separated multistage system exhibit superior nitrogen removal performance to those closer to PND operation. However, the control of pH and management of ammonia washout were still better managed under PND conditions. Given that all trials with the PND system yield competitive carbon requirements for

nitrogen removal compared with published studies, the PND system was found to be superior to the multistage system.

Chapter 8. Conclusions and outlook

8.1 Summary of major conclusions

The purpose of this chapter is to summarise the most important contributions of the research in this thesis to the field of wastewater engineering and research. The overall objective in this study was using physical separation of nitrification and denitrification biomasses into separate chambers for individual optimisation to achieve complete nitrogen removal. This discussion will focus on the key thesis objectives (Section 1.6).

A storage driven denitrification reactor was successfully developed and then combined with an external nitrification reactor to achieve complete nitrogen removal. The observed limitations of the separated biomass multistage system were then overcome by development of the PND reactor. Overall, a reactor system was developed which could treat wastewaters with relatively low C/N ratios (< 4 kg COD/kg N). The low C/N ratios of treatment observed in this study were obtained using synthetic wastewaters with fully biodegradable COD content. Further testing is required of the treatment systems developed herein on the performance with real wastewaters in which the COD content is not expected to be 100% biodegradable.

8.1.1 Likely application of the reactors developed

There is a real need for reactors with the low carbon requirement observed in this study (< 4 kg COD/kg N removed). These reactors would be particularly useful for high nitrogen wastewaters, such as piggery waste, where the COD to nitrogen ratio is comparatively low (~ 6 kg COD/kg N-NH_3 [21] *versus* ~ 11 kg COD/kg N-NH_3 [20]) and the actual concentration of ammonia is high (~ 140 mM NH_3 [21] *versus* ~ 3 mM NH_3 [20]). Such effluents are difficult to treat in traditional systems. It should be noted that the studies contained in this thesis mainly use a synthetic wastewater with 100% biodegradable COD content. It is likely that the performance of the developed systems with real wastewater, with less than 100% biodegradable COD, would require higher COD/N ratios as a result.

8.1.2 Establishment of a storage driven denitrification biofilm

A storage driven denitrification biofilm reactor was established by the alternate feeding of COD rich and nitrate rich feeds. The specifically enriched storage driven denitrification biofilm reactor was kinetically analysed and showed the following features:

- Both glycogen and PHB were detected in the storage driven denitrification biofilm reactor over the course of this study. Reproducible results indicated that PHB was the principle compound produced by the biomass to store the influent acetate in the feed under limited oxygen supply ($k_La < 1.1\ h^{-1}$; $DO < 0.1\ mg\ L^{-1}$) conditions.
- Up to 80% of influent acetate carbon was stored as PHB carbon. The stored PHB was subsequently available for reduction reactions. This amount of storage only

resulted in a very low 'concentration' of PHB in the reactor due to a low feed to mass (F/M) ratio of the reactor. That is, the high biomass level (approximately 50 g) relative to the hydraulic volume of the reactor and the feed volume (1.2 – 1.5 L capacity reactor completely drained for each cycle).

- In contrast to typical microbially catalysed reactions, the uptake of acetate and nitrate reduction followed first order kinetics rather than Michaelis-Menten kinetics. This was explained by diffusion limitation in the thick biofilm.
- Under limited soluble COD supply, the storage driven denitrification reactor was able to achieve > 99% nitrogen removal with less than 4.0 kg COD/kg N-NO_3^-.

8.1.3 Multibiomass wastewater treatment using separated biomass reactors

- The storage driven denitrification reactor was successfully combined with an external nitrification reactor in order to achieve combined nitrification and denitrification of an influent wastewater stream. The high level of conservation of COD for use in denitrification allowed the complete removal of nitrogen at low feed C/N ratios of less than 4.0 kg COD/kg N. SND studies, where conservation of COD has been an integral part of operation and design, still require up to 17 kg COD/kg N removed [29].
- The overall rate of nitrogen removal achieved by the multibiomass system, with SBR or TF based nitrification, was usually observed between 0.4 and 1.1 mM N h^{-1}. This represents an improvement in the overall nitrogen removal rate compared to SND approaches such as that by Gibbs *et al* [41] who observed an overall rate of 0.28 mM N h^{-1}.

- The use of a biofilm for the storage driven denitrification reactor provided an improvement in ammonia removal by reducing the amount of ammonia retained compared to SBR type systems, with as little as 5% ammonia retained from the acetate storage phase.
- The pH drop due to nitrification in the nitrification reactor, under both TF and SBR operation, was observed as a clear shortcoming of the approach of sequential nitrification and denitrification. It caused a substantial reduction of activity of the nitrifiers, and hence the denitrifiers, that relied on substrate supply from the nitrification culture. This problem was addressed by the development of the PND system.

8.1.4 Parallel nitrification and denitrification reactor

The parallel nitrification denitrification (PND) system developed in this project was able to overcome the pH reduction and ammonia retention observed in the multistage system while maintaining high biological nitrogen removal activity. The C/N ratio of removal and rate of nitrogen removal of the multistage reactor was not compromised by conversion to a PND system.

8.1.5 Lack of need for stringent oxygen control for efficient nitrogen removal

Studies of SND indicate that a critical factor in achieving efficient nitrogen removal, in terms of both rate and requirement for carbon, is the control of dissolved oxygen concentration. The optimum oxygen level differs with floc size due to different

diffusion depths for oxygen in large and small flocs. For SND, precise oxygen control is needed and difficult to accomplish. The PND reactor offers a simpler and more robust technology in this regard as it is not the oxygen control that enables nitrification and denitrification to occur at the same time but the separation of biomass into two different reactors.

8.2 Suggested future work

8.2.1 Challenges to direct up scaling of the reactors studied

The system described has only been proven at the laboratory level and used relatively short times to fill the storage driven denitrification reactor as a batch system. Such short fill times are not practical for larger scale operation because of the need of holding tanks to store the wastewater prior to filling and the need of high capacity pumps. For example, in large scale systems the fill time can be as long in practice as the 'react' time, usually resulting in very different reaction profiles than those observed in the laboratory [125].

Short term use of real wastewater showed successful nitrogen removal (0.53 mM h^{-1} overall ammonia removal rate and C/N ratio of removal of 7.6 kg COD/kg N). However, long term operation needs to be verified with respect to the removal of suspended solids and sustained COD storage activity with varying influent COD sources.

8.2.2 Alternative design possibilities for the PND reactor

The PND system could be substantially modified into a single column with a lower anoxic region and an upper oxic region and with upflow hydraulic behaviour using air diffusers applied at the boundary to achieve oxygen delivery (Figure 8.1).

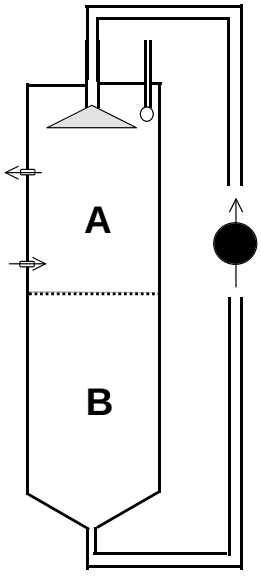

Figure 8.1 Stacked arrangement of the trickling filter nitrification chamber (A) over the submerged storage driven denitrification chamber (B).

As an alternative to separating the biomass as biofilms, the use of very recently improved membrane bioreactors could be used to achieve the separation of biomass and provide a suspended culture.

As wastewater streams are continuous, the operation of a batch process would require storage vessels, which would increase cost. To avoid the use of additional vessels, systems such as SBRs are filled continuously by operation of multiple reactors out of

phase. Out of phase operation is a possibility for PND operation. In addition, it may be possible to operate the PND reactor continuously and this needs to be investigated.

8.2.3 Production of PHB

While the primary focus of this research has been the removal of nitrogen from municipal wastewater streams using sequestered COD (mainly PHB), PHB itself is of research and commercial interest. The high level of conversion of soluble COD into PHB by the storage driven denitrification biofilm may provide a route *via* which the commercial production of PHB can be developed. PHB has potential application as a renewable, biodegradable, thermoplastic. The fact that it can be produced from a waste (wastewater or recycling of PHB based plastic products) by the action of abundantly available naturally occurring organisms (activated sludge) indicates that at least part of the process of producing PHB can be undertaken at low cost. Methods for the low cost extraction, purification and processing of PHB need to be investigated in conjunction with optimisation of the biological process.

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Appendix A. Calculation of theoretical C/N ratios of removal

For industrial relevance, the best measure of C/N ratio for wastewater is the chemical oxygen demand (COD) based ratio. COD is a measure of the oxygen that would be consumed in the oxidation of available carbon. It is more comprehensive than biochemical oxygen demand (BOD), which is the amount of oxygen that a mixed microbial population would consume to oxidise bioavailable carbon, but usually implies a higher amount of carbon than BOD.

Throughout this thesis, the theoretical stoichiometric minimum C/N ratio for removal of nitrate has been referred to in which assimilation of carbon is neglected. In order to calculate this, recall Equation 1.8:

$$5\ CH_3COO^- + 8\ NO_3^- + 9\ H_2O \rightarrow 10\ H_2CO_3 + 4\ N_2 + 13\ OH^-$$

Also note:

$$CH_3COO^- + 2\ O_2 + H^+ \rightarrow 2\ CO_2 + 2\ H_2O$$

	Acetate		Nitrate
Conversion	5 moles CH_3COO^-	*Conversion*	8 moles NO_3^-
× 2 mol O_2 / mol CH_3COO^-	10 moles O_2	*× 1 mol N / mol NO_3^-*	8 moles N
× 32 g mol^{-1}	320 g O_2	*× 14 g mol^{-1}*	112 g N
÷ 1000 g kg^{-1}	0.320 kg O_2	*÷ 1000 g kg^{-1}*	0.112 kg N
× 1 kg COD/ kg O_2	0.320 kg COD		
÷ kg N		2.86 kg COD/kg N-NO_3^-	

A similar calculation can be performed for the reduction of nitrite:

$$3\ CH_3COO^- + 8\ NO_2^- + H_2O \rightarrow 6\ CO_2 + 4\ N_2 + 11\ OH^-$$

Also note:

$$CH_3COO^- + 2\ O_2 + H^+ \rightarrow 2\ CO_2 + 2\ H_2O$$

	Acetate		Nitrite
Conversion	3 moles CH_3COO^-	*Conversion*	8 moles NO_2^-
× 2 mol O_2 / mol CH_3COO^-	6 moles O_2	*× 1 mol N / mol NO_2^-*	8 moles N
× 32 g mol^{-1}	192 g O_2	*× 14 g mol^{-1}*	112 g N
÷ 1000 g kg^{-1}	0.192 kg O_2	*÷ 1000 g kg^{-1}*	0.112 kg N
× 1 kg COD/ kg O_2	0.192 kg COD		
÷ kg N		1.71 kg COD/kg N-NO_3^-	

It has been observed that up to 40% of influent COD may be required directly for growth limiting the amount of COD for denitrification further [18]. An example calculation incorporating this growth requirement is shown below:

	Acetate		Nitrate
Feed amount	100 moles CH_3COO^-		
Less 'growth' (40%) loss	60 moles CH_3COO^-		
Conversion		*Conversion*	
		× 8/5 mol NO_3^- / mol CH_3COO^-	96 moles NO_3^-
× 2 mol O_2 / mol CH_3COO^-[36]	200 moles O_2	*× 1 mol N / mol NO_3^-*	96 moles N
× 32 g mol^{-1}	6400 g O_2	*× 14 g mol^{-1}*	1344 g N
÷ 1000 g kg^{-1}	6.40 kg O_2	*÷ 1000 g kg^{-1}*	1.34 kg N
× 1 kg COD/ kg O_2	6.40 kg COD		
÷ kg N		4.76 kg COD/kg N-NO_3^-	

[36] Amount of COD considered in this section of the calculation must be based on the influent feed amount, not the amount used for storage and subsequent reduction of nitrate

Since acetate was used as the main electron donor source for the reactors tested in this thesis, the C/N ratio of removal (Section 2.2.6) determined in each experiment was found by converting acetate into COD as shown above. As a result, the COD values quoted in this thesis mainly refer to biodegradable COD.

Appendix B. Estimation of carbon balance

Due to the difficulties in representative sampling of the biomass in the storage driven denitrification biofilm, an alternative route was sought to estimate a carbon balance. The storage driven denitrification reactor was fed a normal acetate feed during acetate uptake phase, followed by an excess of nitrate during the denitrification phase (Section 2.3.3). The following is an outline of the calculation method for that experiment which also serves to demonstrate the general form of estimating amount of carbon lost *via* oxidation in other experiments.

Table B.1 Calculation of the carbon balance from the excess nitrate fed experiment (Section 2.3.3).

Acetate uptake phase (AUP):		Denitrification phase (DNP):	
Acetate removed from solution	2.02 mM	*Nitrate removed from solution*	1.97 mM
Amount of electrons available from acetate	16.17 mM	*Electrons donated to nitrate*	9.83 mM
Average OUR during AUP	8.45 mg L^{-1} h^{-1}	*Nitrite remaining in solution*	0 mM
Duration of AUP	97.22 min	*Electrons donated to convert nitrate to nitrite*	0 mM
ie oxygen transferred during AUP	13.69 mg L^{-1}	*Nitrous oxide remaining in solution*	8.14×10^{-4} mM
	0.43 mM	*Electrons donated to convert nitrate to nitrous oxide*	1.63×10^{-3} mM
Number of electrons donated to oxygen[37]	1.71 mM	*Total electrons accepted by nitrogen*	9.83 mM
Number of electrons donated by acetate	1.71 mM	*Average OUR during DNP*	8.17 mg L^{-1} h^{-1}
Amount of acetate oxidised	0.21 mM	*Duration of DNP*[38]	180.00 min
% of acetate oxidised	**11%**	*ie oxygen transferred during DNP*	24.52 mg L^{-1}
			0.77 mM
		Number of electrons donated to oxygen	3.07 mM
		Number of electrons consumed during DNP	12.90 mM
		% electrons stored based on nitrate reduction during DNP	61%
		% electrons stored based on oxygen consumption during DNP	19%
		Total % electrons from acetate stored	**80%**
% of acetate assimilated	**9%**		

[37] See Table B.2 for electron equivalents of each species involved.

[38] Duration of DNP considered only until nitrate reduction assumed to be ceased.

Table B.2 Electron equivalents involved in the storage driven denitrification reactor.

Species	Electrons transferred per mole
Oxygen	4
Nitrate	5
Nitrite	3
Nitrous oxide	2
Acetate	8

Appendix C. Kinetic analysis of nitrogen removal

Enzyme mediated processes tend to obey Michaelis-Menten kinetics of the form:

$$V = \frac{V_{\max}[S]}{k_M + [S]}$$

which can be analysed *via* the Lineweaver-Burk plot of 1/V *vs* 1/[S] yielding a straight line with slope k_m/V_{max} and intercept $1/V_{max}$. The raw data used for analysis of the kinetics of nitrate removal (Section 2.3.1) are shown below (Figure C.1). The subsequent Lineweaver-Burk plot (Figure C.2) indicated good agreement with Michaelis-Menten kinetics (R^2 = 0.99). However, the predicted data based on the derived V_{max} and k_m do not match well with the actual data (Figure C.3).

It is possible that the observed kinetics are limited by diffusion of the substrate to the bacteria in the biofilm. Diffusion is a first order process. A plot of the natural logarithm of the substrate concentration *vs* time will yield a straight line with slope, k, if first order kinetics are obeyed (Figure C.4). Again the resulting predicted data do not match well with the actual data (Figure C.3). The shape of the curve of actual substrate disappearance is more closely matched to that for diffusion limited kinetics than Michaelis-Menten kinetics (Figure C.4).

The analysis was repeated for nitrite removal. Raw data (Figure C.5) were analysed using a Michaelis-Menten model (Figure C.6) and first order model (Figure C.7). The derived kinetic parameters were used to predict the loss of nitrite (Figure C.8).

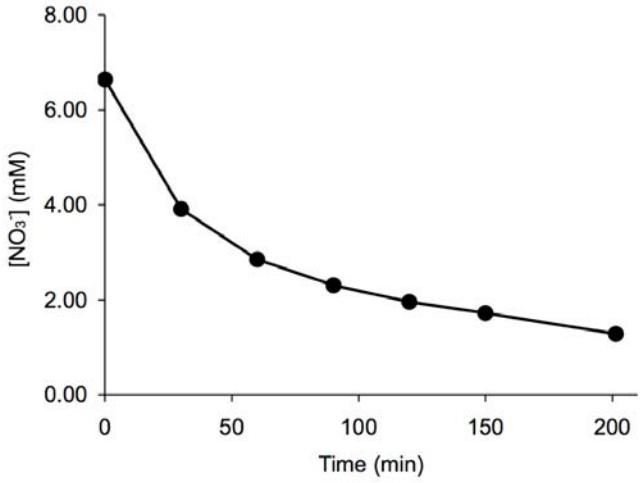

Figure C.1 Change in nitrate concentration during nitrate reduction phase of storage denitrification reactor operation.

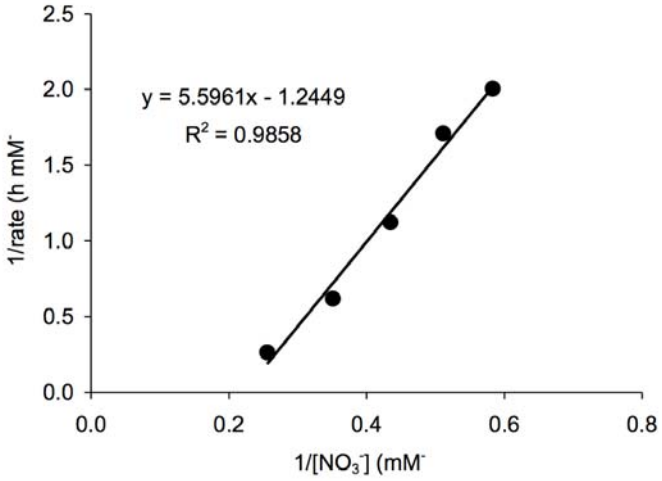

Figure C.2 Lineweaver-Burk plot of nitrate data from Figure C.1.

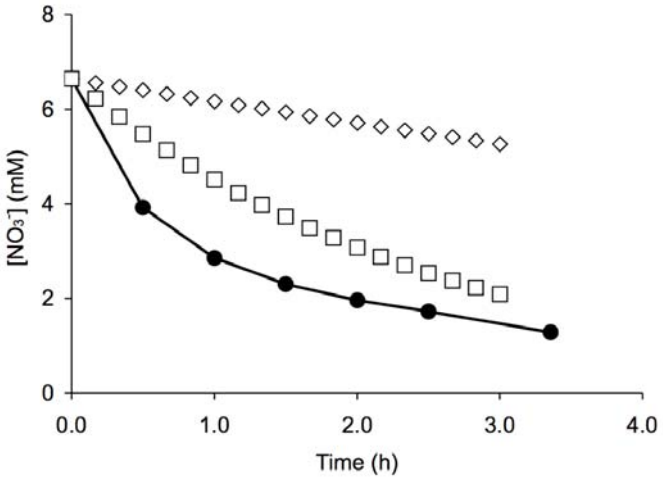

Figure C.3 Agreement between actual nitrate data (●) and modelled data derived *via* Michaelis-Menten kinetics (◇) and first order diffusion controlled kinetics (□).

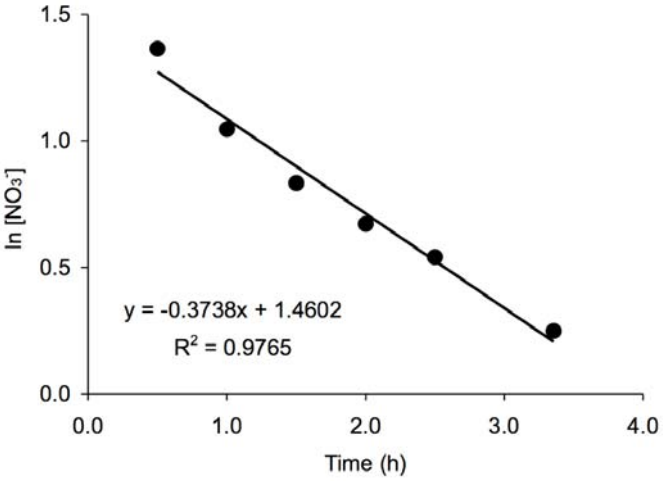

Figure C.4 First order rate constant derivation for nitrate reduction of data from Figure C.1.

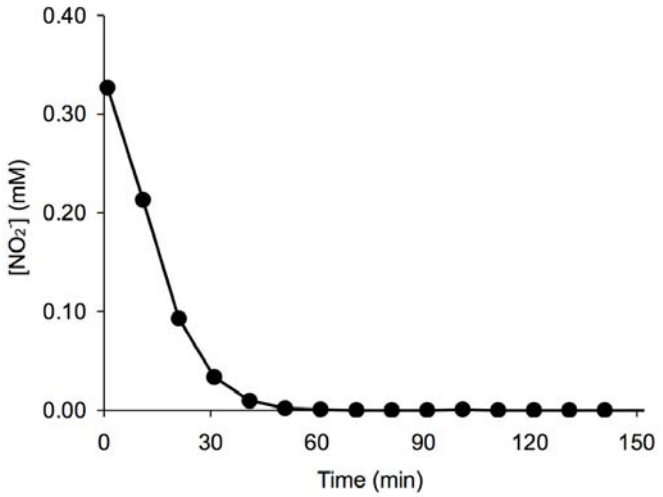

Figure C.5 Change in nitrite concentration during nitrite reduction phase of storage denitrification reactor operation.

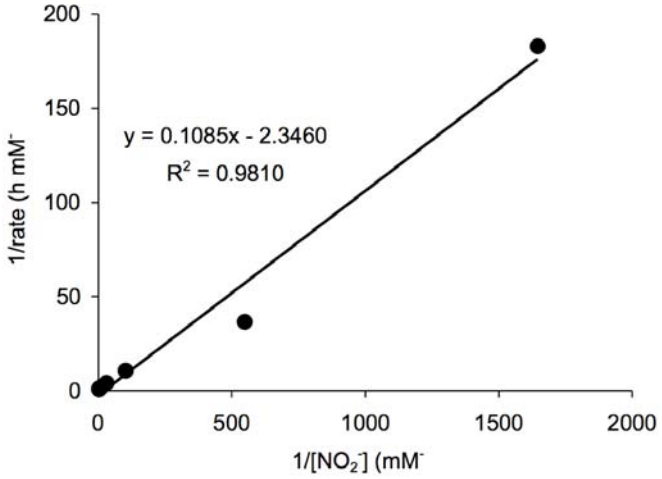

Figure C.6 Lineweaver-Burk plot of nitrite data from Figure C.5.

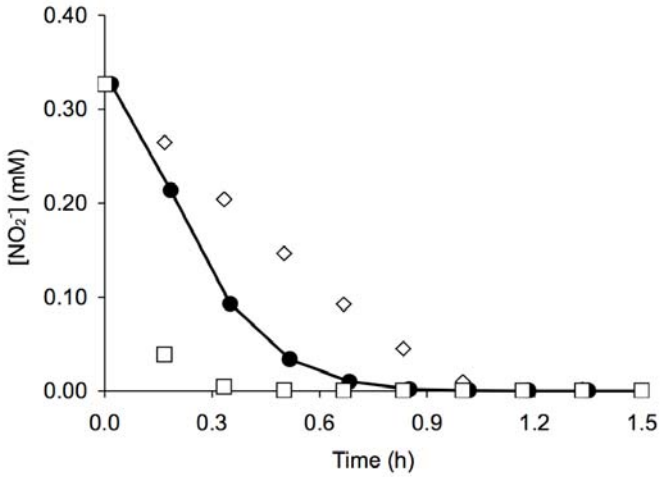

Figure C.7 Agreement between actual nitrite data (●) and modelled data derived *via* Michaelis-Menten kinetics (◇) and first order diffusion controlled kinetics (□).

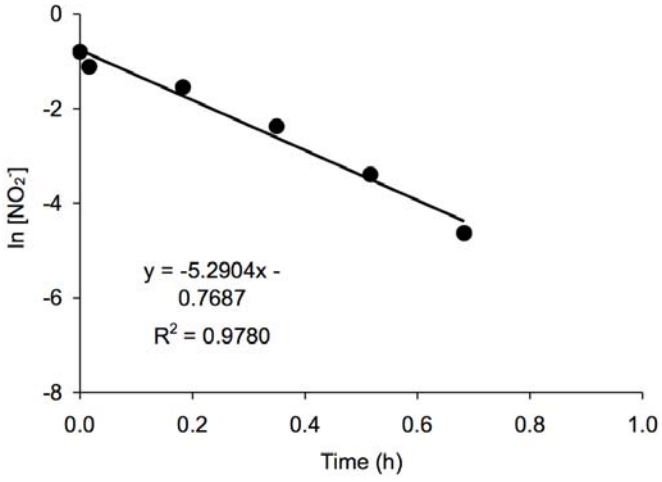

Figure C.8 First order rate constant derivation for nitrite reduction of data from Figure C.5.

Appendix D. Carbon mass balance in constant acetate feed experiment

An experiment was conducted to attempt to quantify the maximum amount of COD storage that could occur in the storage driven denitrification biofilm reactor by feeding constant acetate over a sustained length of time (> 10 hours). Due to the high amount of biomass present in the reactor (~ 50 g volatile solids and ~ 100 g total solids (Appendix F), the yield of PHB expressed as a percent of dry cell mass was very low. The following is an attempt to reconcile the apparent mass balance variation.

Conversion	Result
Total removed from solution	17 mM CH_3COO^-
× 8 mol e^- / mol CH_3COO^-	136 mM e^-
÷ 18 mol e^- / mol PHB	7.56 mM PHB
× 86 g mol^{-1} PHB	650 mg PHB
÷ 50 000 mg biomass/L reactor	0.013 mg PHB/mg biomass
× 100	1.3 % PHB by mass

The actual observed yield of PHB was 1.2% of dry cell mass (Section 2.3.5), which is in good agreement with that predicted above.

Appendix E. Determination of the fate of ammonia in the storage driven denitrification biofilm reactor

Ammonia was reproducibly observed to disappear during acetate uptake and appear during denitrification in the storage driven denitrification biofilm. This trend was maintained throughout operation alone (*eg* Figure 2.4) and in conjunction with nitrifiers in the multistage system (*eg* Figure 4.14). In addition, during parallel nitrification denitrification (PND) operation, where the acetate uptake phase is identical to that in the storage driven denitrification process and the multistage system, ammonia was also observed to decrease (*eg* Figure 5.4). The rapid rate at which ammonia appeared and disappeared was sometimes in excess of the nitrification rate observed in the trickling filter reactor (*eg* Figure 4.14). In each phase a stable concentration of ammonia was usually reached within 30 minutes.

The appearance of ammonia during denitrification limited the nitrogen removal performance of the storage driven denitrification and multistage systems while the disappearance during acetate uptake appeared to contribute to nitrogen removal. Since the effect was substantial, with up to 40% of influent ammonia disappearing during acetate uptake, it requires explanation so that the possible contribution to nitrogen removal could be estimated. The accumulated data of ammonia behaviour for the storage driven denitrification biofilm in all reactor configurations was used to elucidate a possible explanation.

E.1 Additional laboratory data on the behaviour of ammonia

The loss of significant amounts of influent ammonia (up to 40%) during the acetate uptake phase of the storage driven denitrification system coincident with acetate uptake suggests that the loss of ammonia was related to the acetate uptake process, most likely the result of assimilation. However, small scale tests undertaken to confirm the mass balance (Section 2.3.3) yielded a release of ammonia rather than an uptake as in the laboratory scale reactor (Figure E.1). The need for ammonia to be supplied concurrently with acetate for assimilation was probed in additional small scale tests in which ammonia was not supplied. The absence of ammonia appeared to have no impact on the metabolism of acetate in the tests (Figure E.2). PHB production from acetate was similar under conditions where ammonia was supplied (0.31 ± 0.08 mmole PHB/mmole acetate) and not supplied (0.34 ± 0.10 mmole PHB/mmole acetate).

E.2 Potential explanations for ammonia uptake

E.2.1 Assimilation

There are a number of possible explanations for the fate of ammonia in the storage driven denitrification biofilm reactor, both biological and physicochemical, some more likely than others. In the mass balance experiments based on addition of excess nitrate (Section 2.3.3), the observed loss of ammonia was 0.5 mM, 22% of the total influent nitrogen. Based on a typical empirical formula for biomass, $C_5H_7NO_2$ [122], the

corresponding carbon that would be required for growth accounts for 60% of acetate supplied. Although this amount of assimilation has been reported previously, [126], it was unlikely that this much growth occurred in the storage driven denitrification reactor where up to 80% storage was observed. Assimilation could also be ruled out as a dominant process by the fact that ammonia loss during acetate uptake was not related to acetate metabolism as acetate loss and production of stored COD occur regardless of the presence of ammonia (Section E.1). Ammonia removal still occurred when acetate was not present (Figure E.3) while acetate uptake occurred when ammonia was not present (Figure E.2). Therefore, if ammonia was substantially metabolised, it was likely to be a separate process from acetate metabolism.

E.2.2 Oxidation

During acetate uptake, the removal of ammonia could be related to the oxidation of ammonia by nitrification. It is unlikely this was a significant contributor to ammonia loss as there was very low oxygen supply to this reactor ($k_La < 1.1\ h^{-1}$) and no corresponding release of nitrate or nitrite was observed (Figure 2.4). It is possible that any nitrate or nitrite would be consumed by the bulk heterotrophic biomass present prior to reaching the recycle vessel for detection. Since no reduction in denitrification capacity was observed in the next phase, this was unlikely. As discussed earlier, the supply of oxygen for oxidation of acetate was insufficient to account fully for the energetics involved and a loss of oxygen through nitrification would further complicate this, even if the oxidised nitrogen species were used as alternative electron acceptors to oxygen.

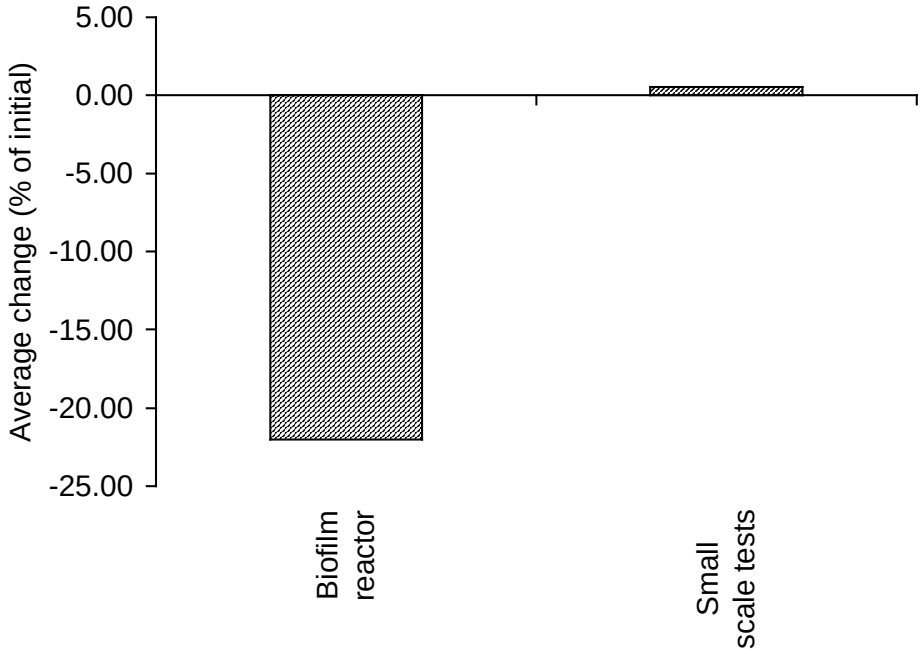

Figure E.1 Average observed change in behaviour of ammonia in the storage driven reactor and the small scale tests (Section 2.3.3).

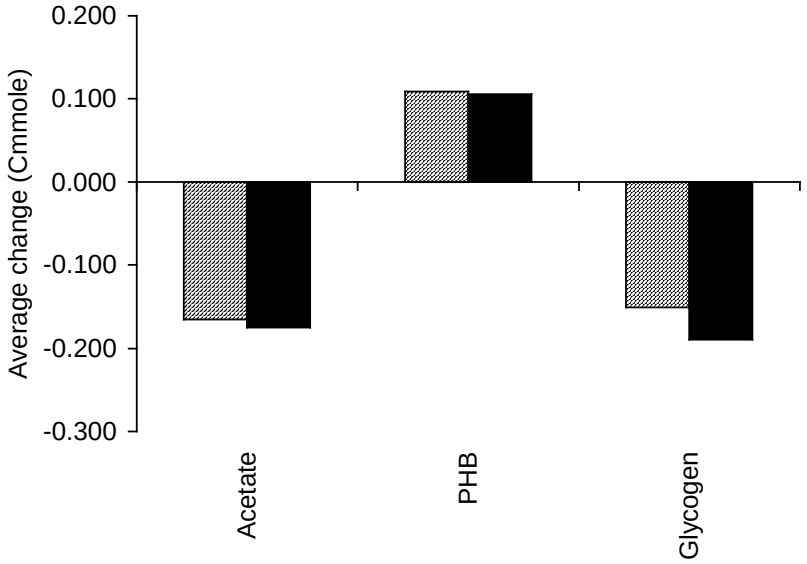

Figure E.2 The effect of ammonia on the change in metabolites (Cmmole) in storage driven denitrification biomass removed from bioballs by washing and incubation (180 minutes) in a 20 mL anaerobic shake flask at a biomass concentration of 7.7 g/L with 4.50 mM acetate medium (Section 2.2.1). Ammonia not supplied (striped columns); ammonia supplied at 2.43 mM (solid columns).

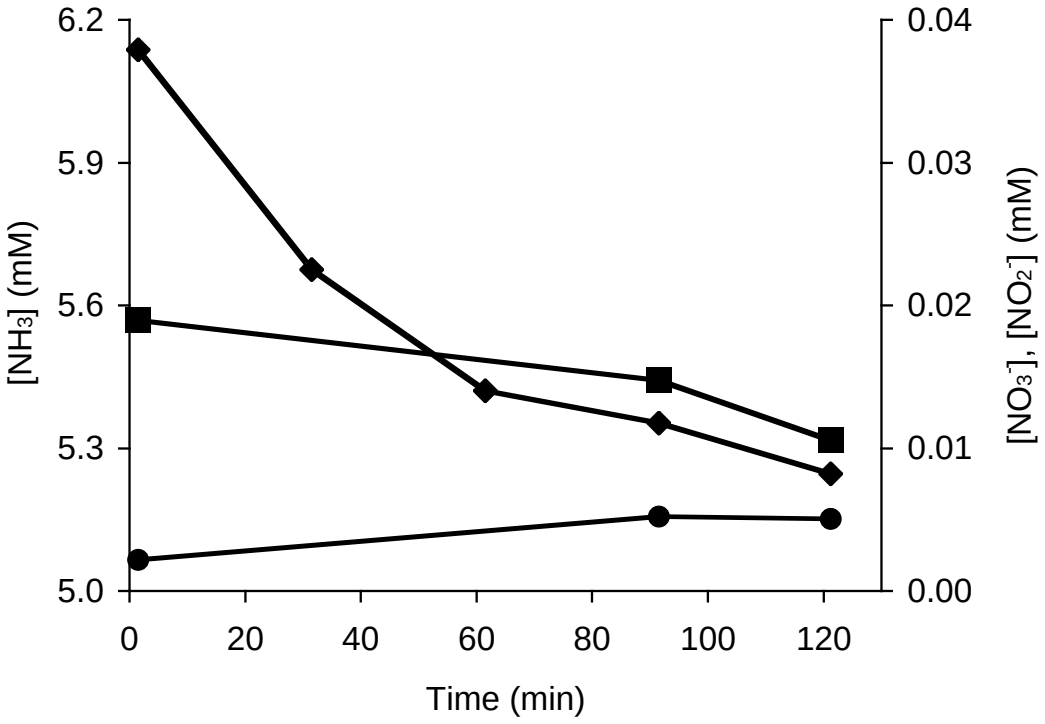

Figure E.3 The effect of a no acetate feed on ammonia uptake during the acetate uptake phase of the storage driven denitrification reactor. Feed: ammonia 6.1 mM. Ammonia (◆), nitrate (■) and nitrite (●).

An alternative ammonia removal mechanism is that of anammox [127], the ANaerobic AMMonium OXidation producing elemental nitrogen gas according to the equation below:

$$1\ NH_4^+ + 1.3\ NO_2^- \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 2\ H_2O \qquad \text{Equation E.1}$$

As can be seen, this reaction requires the supply of nitrite, which was not detected during acetate uptake. Some modifications on the basic anammox process, like CANON [128, 129] and OLAND [130, 131], introduce small quantities of oxygen so that nitrification and anammox can occur simultaneously (Equation E.2). As this process produces nitrate, a normal fed cycle with influent ammonia of 2.99 mM from which approximately 20 – 30% is lost, should yield up to 0.12 mM of nitrate. No nitrate was detected and no subsequent reduction in denitrification capacity from stored substrate was observed.

$$1\ NH_4^+ + 0.85\ O_2 \rightarrow 0.435\ N_2 + 0.13\ NO_3^- + 1.3\ H_2O + 1.4\ H^+ \qquad \text{Equation E.2}$$

In addition, an experiment with minimal oxygen supply fed with normal synthetic wastewater with additional nitrite did not show any difference in ammonia removal (Figure E.4). If a significant population of anammox bacteria were present, an increase in the amount of ammonia lost would be detected. For this experiment, as the amount of ammonia removed was significant (> 3mM), production of nitrate should have been detectable. The maximum that would be observed would be 0.4 mM. Only 50% of this was observed.

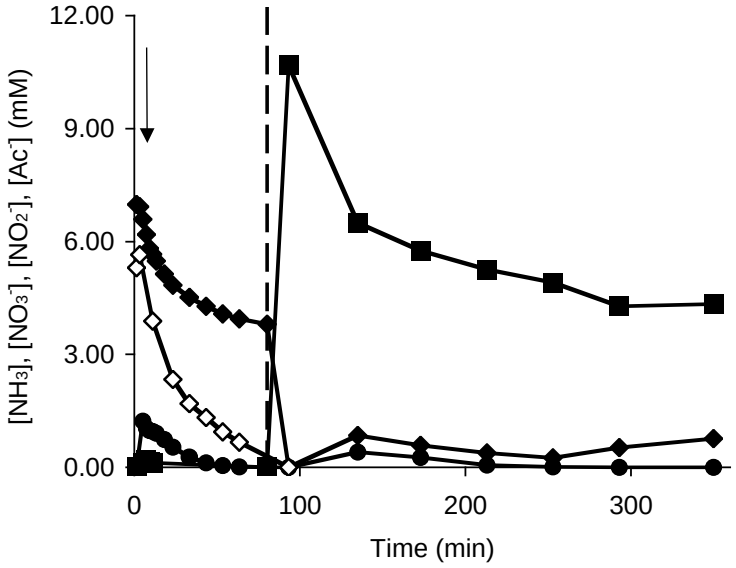

Figure E.4 Effect of a spiked nitrite (to 1.2 mM at 3 minutes into acetate uptake phase indicated by arrow) on the removal of ammonia during the acetate uptake phase and removal of nitrate during the denitrification phase of the storage driven denitrification reactor. Feed during acetate uptake phase: 5.3 mM Acetate and 7.0 mM ammonia, C/N ratio 3.5 kg COD/kg N; Feed during denitrification phase: 11 mM nitrate. Ammonia (◆), nitrate (■), nitrite (●) and acetate (◇). Vertical dotted line indicates change of phase from acetate uptake phase (90 minutes) to denitrification phase (270 minutes).

The subsequent nitrate removal phase seemed to be unaffected by the addition of nitrite which could indicate that there was anammox activity as there was no substantially reduced availability of electron donor. However, this was unlikely to be a significant

contributor during normal operation as the supply of nitrite in acetate uptake was negligible. In addition, it has been shown that the typical C/N ratio of the synthetic wastewater used for this study is in excess of that required, allowing additional nitrogen removal when the supply of carbon is insufficient based on luxury storage of excess carbon (Section 5.3.5). This indicated that anammox was unlikely to be a significant contributor to ammonia removal, even if a population of anammox organisms is present in the biofilm.

E.3 Appearance of ammonia during the denitrification phase

For appearance of ammonia during the denitrification phase, the main possible metabolic contributors would be decay of biomass yielding free ammonia from the breakdown of proteins and the dissimilatory reduction of nitrate to ammonia. The latter was not observed since the appearance of ammonia was detected even if nitrate, or nitrite, was not present (Figure E.5).

Attempts to isolate the principle microorganism in the culture responsible for the storage of acetate have yielded large numbers of amoeba (Figure E.6). Grazing by amoeba may cause the appearance of ammonia due to the degradation of the heterotrophic biofilm biomass. The release of ammonia through decay of biomass could also be related to endogenous respiration of the heterotrophic biomass [132]. As the culture had reached a steady state, determined by reproducible and stable activity levels, the estimation of the release of ammonia through this process would also yield an

estimation of the concurrent rate of microbial growth and hence nitrogen and carbon assimilation.

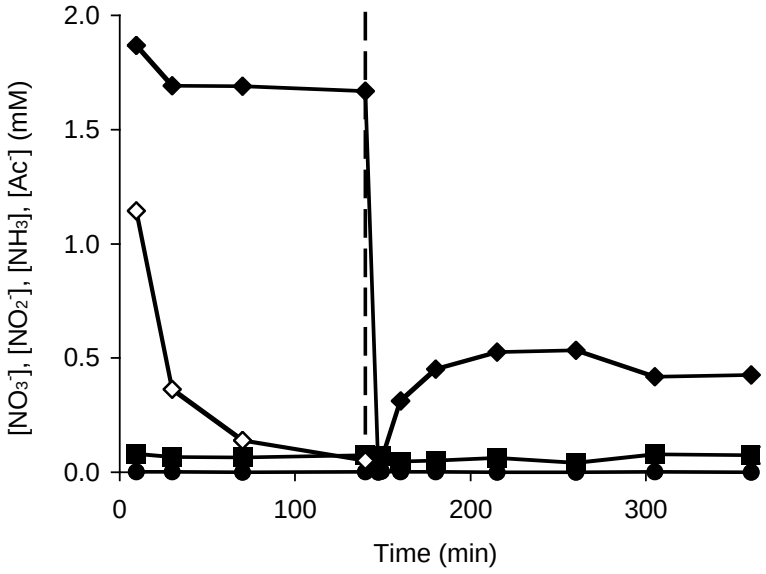

Figure E.5 Observation of the appearance of ammonia during the denitrification phase where no nitrate or nitrite was fed. Ammonia (◆), nitrate (■), nitrite (●) and acetate (◇). Feed during acetate uptake phase: 2.0 mM Acetate and 1.9 mM ammonia, C/N ratio 4.8 kg COD/kg N; Feed during denitrification phase: 0.06 mM nitrate. Vertical dotted line indicates change of phase from acetate uptake phase (140 minutes) to denitrification phase (220 minutes).

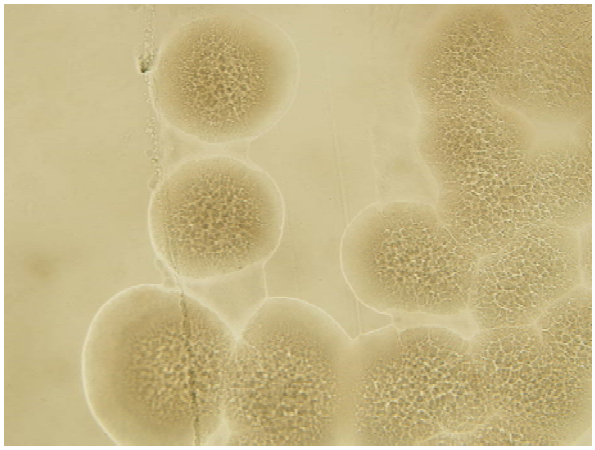

Figure E.6 Amoebic cysts observed on agar plates grown from samples of the storage driven denitrification biofilm. Magnification 100 × 1.4. Image courtesy of Rajat Hans 2006.

E.4 Physical adsorption of ammonia the biofilm matrix

The most significant contribution to the loss of ammonia in acetate uptake and gain in denitrification is likely to be a physical process as the metabolic reactions described above are inadequate to fully account for the fate of ammonia. In particular, because the culture appears to be close to a monoculture microscopically, it is unlikely that significant populations of nitrifiers or anammox bacteria would remain undetected.

Evidence for a bulk physical equilibration can be taken from a number of experimental results. The first was that the substantial loss of ammonia in acetate uptake and gain in denitrification was significantly reduced after cleaning the bioreactor. Cleaning involved the mixing of the bioballs and removing biofilm in the tubing and therefore disruption of the extracellular polymeric substances (EPS) in the biofilm matrix. Similarly, in the small scale mass balance experiments (Section E.1) disruption of the biomass coincided with a release of ammonia into the bulk liquor during incubation by an average of 0.5%. As it was likely to be the EPS material into which the ammonia equilibrated, the loss or disruption of EPS would be expected to affect the ammonia adsorption of the culture.

Furthermore, in cycles where the influent ammonia concentration to the acetate uptake phase was very low or negligible, the ammonia concentration generally increased during acetate uptake, analogous to the gain in ammonia observed in denitrification (Figure E.7), which may indicate physical retention of ammonia from the previous denitrification phase.

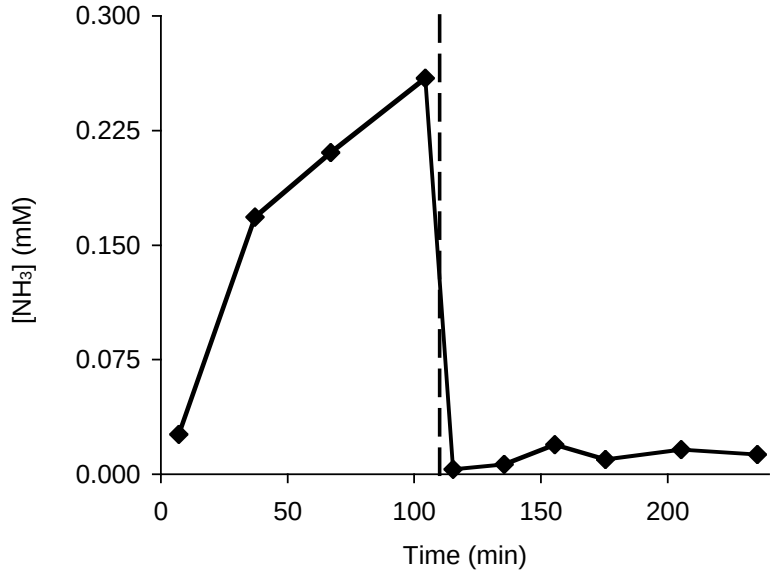

Figure E.7 The effect of a very low influent ammonia concentration to the acetate uptake phase on the effluent ammonia concentration from the acetate uptake phase. Vertical dotted line indicates change of phase from acetate uptake phase (110 minutes) to denitrification phase (130 minutes).

Reasonably strong linear relationships were evident between the influent feed ammonia concentration to the acetate uptake phase and that exiting the phase (Figure E.8; Slope = 0.8273 and R^2 = 0.8005) and also between the acetate uptake effluent ammonia concentration and the denitrification phase effluent ammonia concentration (Figure E.9; Slope = 0.2823 and R^2 = 0.8273). This also supports a bulk physical adsorption or dilution process.

The comparatively slow mixing in the biofilm system could explain the measurable rate of appearance and disappearance of ammonia in each phase. The establishment of a plateau concentration of ammonia in each case also indicates that each phenomenon may due to a physical equilibrium rather than a metabolic process where the gel phase of a biofilm is in equilibrium with the bulk liquor phase (Figure E.10).

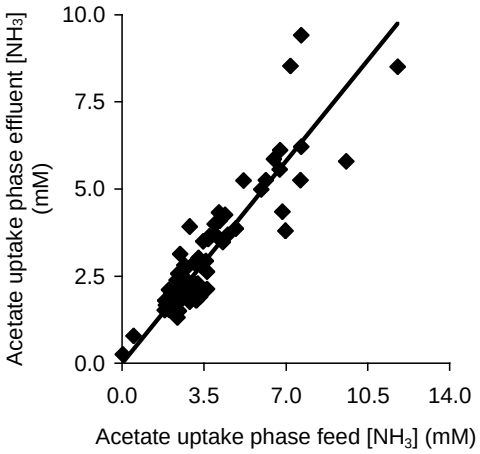

Figure E.8 Apparent linear variation of the ammonia concentration in the effluent of the acetate uptake phase with influent ammonia concentration to the acetate uptake phase in the storage driven denitrification reactor. Slope = 0.8273, R^2 = 0.8005.

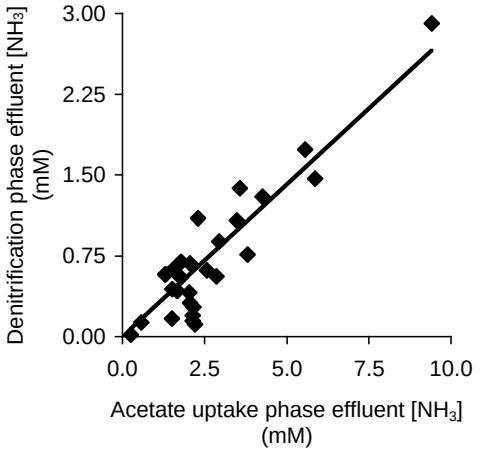

Figure E.9 Apparent linear variation of the ammonia concentration in the denitrification phase effluent with the acetate uptake phase effluent in the storage driven denitrification reactor. Slope = 0.2823, R^2 = 0.8273.

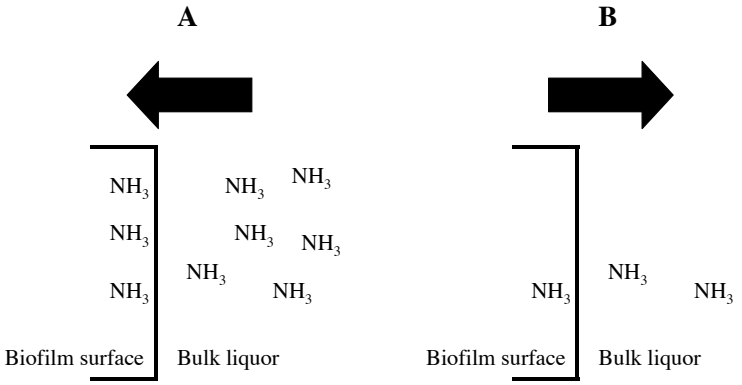

Figure E.10 Model of physical equilibration of ammonia between the bulk liquor and the biofilm matrix in the storage driven denitrification biofilm during the acetate uptake phase (A) and the denitrification phase (B). In the acetate uptake phase, the concentration of ammonia in the bulk is high and so the amount adsorbed into the matrix increases until equilibrium is reached. In the denitrification phase, where the bulk liquor ammonia concentration is negligible, adsorbed ammonia is released until equilibrium is reached. Arrows indicate net movement of ammonia in each phase.

E.4.1 Quantification of adsorption and assimilation of ammonia

If the behaviour of ammonia in this reactor was solely due to a physical process, then the amount missing during acetate uptake should match that gained during denitrification. However, only 17% (1 – slope of Figure E.8) disappears during acetate uptake while 28% (slope of Figure E.9) appears during denitrification, indicating an additional appearance of 11% which must be accounted for. The assumption of a bulk physical equilibration process is used as a basis for analysis.

Using the simple model described above (Figure E.10), it was possible to analyse the accumulated data for ammonia behaviour in the storage driven denitrification biofilm. It should be noted that the following attempt to explain and quantify the role of ammonia in the storage driven denitrification culture is tentative and suggestive of a trend rather than a complete mass balance given the very wide range of experimental conditions included in the analysis that follows.

As dilution is a very simple process to model, this was used as a basis on which to analyse the data. In order to model the process and create a prediction to compare to the actual values, it was necessary to choose one phase as a basis in which the ammonia change was solely due to a physical equilibration. This was done for each phase and then used to predict the outcome of the alternative phase. The results are very similar and both approaches will be discussed below.

E.4.2 Assumed assimilation during the acetate uptake phase

In order to estimate the proportion of ammonia that is adsorbed, the denitrification phase, which has simpler assumptions, was considered first. Following this estimation, the same process was completed in reverse considering the acetate uptake phase first instead. The assumptions for the denitrification phase are:

1. A 'void' volume is retained as part of the biofilm. It is not clear if the retention is due to an equilibrium with an actual void volume or adsorption onto the biofilm surface. Given that biofilms are predominantly water, up to 95% [71, 133], the existence of a void volume is a reasonable assumption. The concentration of ammonia in the void is assumed to be the same as the effluent ammonia concentration from the preceding acetate uptake phase.
2. The influent feed for denitrification does not contain any ammonia. This is not truly an assumption as the feed is defined, prepared and measured with no ammonia.
3. Ammonia that appeared in the denitrification phase was the result of a physical equilibration between the void volume, from assumption 1, and the influent ammonia free feed, from assumption 2.
4. All of the predictions used real data where available rather than compound error by using predicted data.

From these assumptions, the system was modelled using a simple dilution equation as follows:

$$c_1V_1 = c_2V_2 + c_3V_3 \qquad \text{Equation E.3}$$

where condition 1 refers to the effluent from the denitrification phase, condition 2 is the void and condition 3 is the feed. As the feed ammonia concentration is 0, condition 3 can be eliminated from the equation thus:

$$c_1 V_1 = c_2 V_2 \qquad \text{Equation E.4}$$

If the feed volume is *V* and the void volume is *v* then the final volume, for condition 1, will be *V* + *v*. Also, the relationship between the effluent from denitrification, c_1, and the effluent from the preceding acetate uptake phase, c_2 is given from the slope, *b*, of the curve in Figure E.9. As a result, Equation E.4 becomes:

$$bc_2(V + v) = c_2 v \qquad \text{Equation E.5}$$

from which c_2 can be eliminated. The equation can be rearranged to find the void volume based on *b*:

$$v = zV \qquad \text{where} \quad z = \frac{b}{1 - b} \qquad \text{Equation E.6}$$

The same manipulation can be done using each experimental data point as follows to calculate the average value for *v* over the accumulated data.

$$v = zV \qquad \text{where} \quad z = \frac{c_1}{c_2 - c_1} \qquad \text{Equation E.7}$$

The value for *z* based on the slope data was found to be 0.393 while that from the average of the data points was 0.425. The value derived from the slope was used for subsequent calculations as it agrees well with that found by considering the acetate uptake phase (see below). The value of *z* can be substituted into Equation E.4 to find the denitrification effluent ammonia concentration, c_1:

$$c_1 = \frac{c_2 z}{1 + z} \qquad \text{Equation E.8}$$

The relationship between predicted and actual denitrification effluent ammonia concentration indicates good agreement (Figure E.11; Slope = 0.9295; R^2 = 0.7770) with a small underestimation.

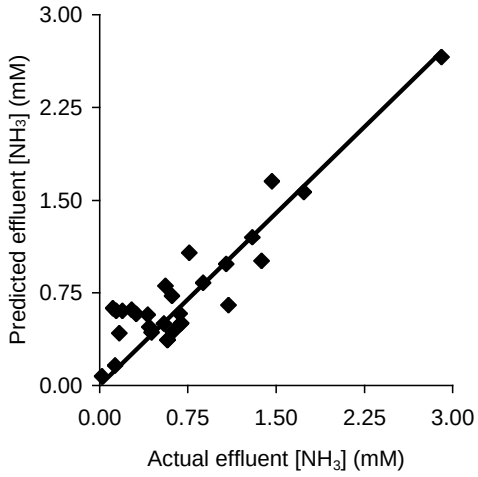

Figure E.11 Ammonia concentration in the effluent of the denitrification phase as measured compared to predicted. Slope = 0.9295, R^2 = 0.7770.

The value for *z* can be applied in turn to the acetate uptake phase to check the extent of adsorption of ammonia in that phase. The assumptions applied are similar to those for the denitrification phase above:

$$c_1V_1 = c_2V_2 + c_3V_3 \qquad \text{Equation E.9}$$

where condition 1 refers to the effluent from the acetate uptake phase, condition 2 is the void and condition 3 is the feed. From the relationship established based on the denitrification phase, this can be substituted as follows:

$$c_1(V + zV) = c_2zV + c_3V \qquad \text{Equation E.10}$$

V can be eliminated from the equation and rearranged to give the acetate uptake phase effluent based on the feed and denitrification effluent ammonia concentrations:

$$c_1 = \frac{c_2z + c_3}{z + 1} \qquad \text{Equation E.11}$$

The comparison of actual and predicted effluent from the acetate uptake phase based on this relationship does not account for all of the ammonia lost (Figure E.12; Slope = 0.9714, R^2 = 0.8781). Approximately 3% of the ammonia is not accounted for in the

effluent from the acetate uptake phase and an additional 7% is unaccounted for in the denitrification phase.

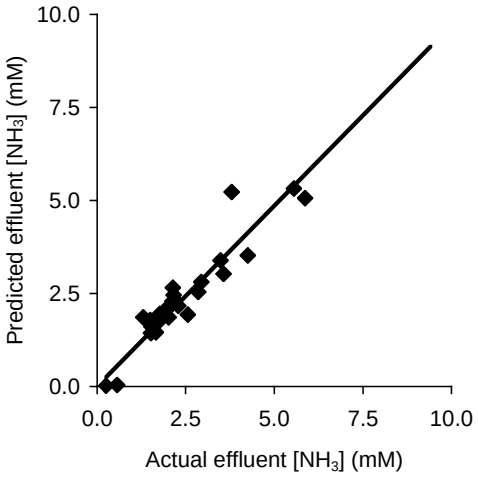

Figure E.12 Ammonia concentration in the effluent of the acetate uptake phase as measured compared to predicted. Slope = 0.9714, R^2 = 0.8781.

It was expected that the prediction based on physical equilibration would yield and overestimation of ammonia in the second phase assessed, here the acetate uptake phase. An overestimation would clearly indicate the assimilation of ammonia. However, the underestimation links with the presence of amoeba discussed earlier as that may increase the amount of ammonia released. As the culture was in a steady state, the estimation of gain of ammonia due to biofilm degradation should match the loss due to assimilation. Therefore the underestimation can still be used to apportion ammonia to growth and adsorption processes.

As the general accepted formula for biomass is $C_5H_7NO_2$, any ammonia taken up for assimilatory purposes would need to have concurrent carbon assimilation of five times that amount, or approximately 2.5 times that amount of acetate, as it contains two moles

of carbon atoms per mole. The percentage of carbon for assimilatory purposes of that fed overall is highly variable (mean = 14.1% and standard deviation = 11.4%). However, the percentage of acetate assimilated varies strongly linearly with the N/C ratio of the feed (Figure E.13; slope = 50.85 and R^2 = 0.9489) based on this calculation.

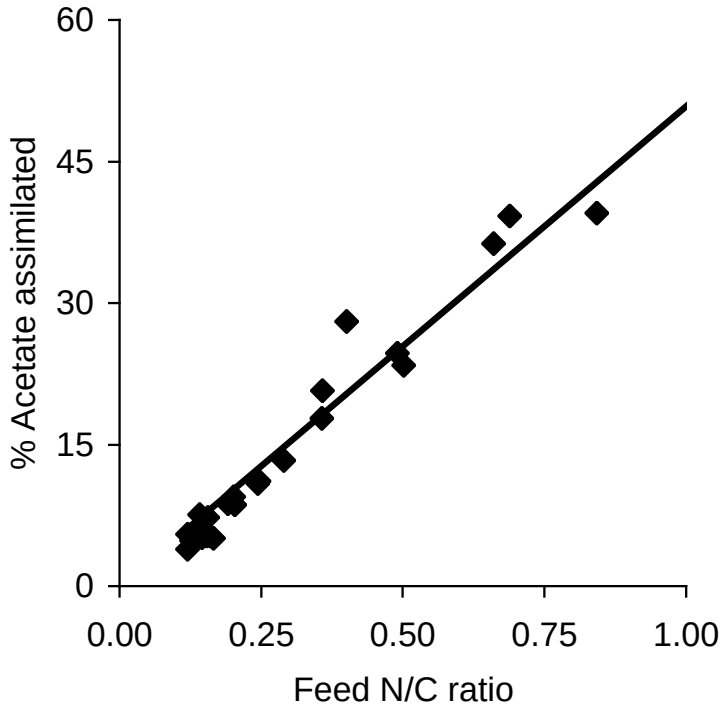

Figure E.13 Variation in estimated percentage of influent acetate used for biomass production with influent N/C ratio. Slope = 50.8, R^2 = 0.9489.

It is known that the optimal conditions for accumulation of intracellular PHB are those that supply an imbalance of nutrients for growth such as an increasing C/N ratio of feed [28]. The variation in estimated assimilated carbon with N/C ratio supports this. A high N/C ratio encourages growth and hence assimilation of nitrogen rather than storage of the carbon for denitrification.

E.4.3 Assumed assimilation during the acetate uptake phase

The above model can be applied in reverse by considering the acetate uptake phase first. If this is done, the values for *z* vary considerably between that based on the slope of Figure E.8, *a*, and the slope of Figure E.9, *b* (Equation E.12) and that based on the average of the individual experiments (Equation E.13).

$$v = zV \qquad \text{where} \quad z = \frac{1-a}{a-ab} = 0.291 \qquad \text{Equation E.12}$$

$$v = zV \qquad \text{where} \quad z = \frac{c_3 - c_1}{c_1 - c_2} = 0.383 \qquad \text{Equation E.13}$$

Since that value for *z* determined by individual data points is in closer agreement with that for the denitrification phase calculation, this value was used for subsequent calculations.

Again, both phases show a small amount of ammonia unaccounted for. The predicted acetate uptake effluent (Figure E.14; Slope = 0.9782, R^2 = 0.8392) again has approximately 2% missing while the predicted denitrification phase effluent (Figure E.15; Slope = 0.9124, R^2 = 0.7770) has approximately 9% missing. The estimated amount of acetate that would be assimilated again varies linearly with the feed N/C ratio (Figure E.16; Slope = 50.04, R^2 = 0.9212), in good agreement with that found in the previous estimation (Figure E.13).

In general, the majority of ammonia assimilation appeared to occur during denitrification (72 – 80%). This was an expected result given that growth is more likely to occur where bacteria have an abundance of both electron acceptor and donor. The denitrification phase more closely fits these criteria than the acetate uptake phase.

Although it is known that a small amount of oxygen was available during the acetate uptake phase, it was insufficient to account for growth in that phase.

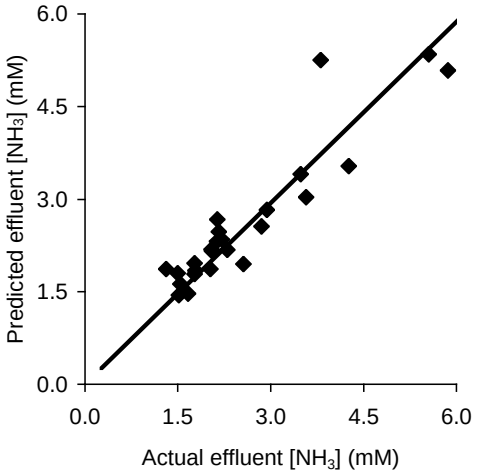

Figure E.14 Ammonia concentration in the effluent of the acetate uptake phase as measured compared to predicted. Slope = 0.9782, R^2 = 0.8392.

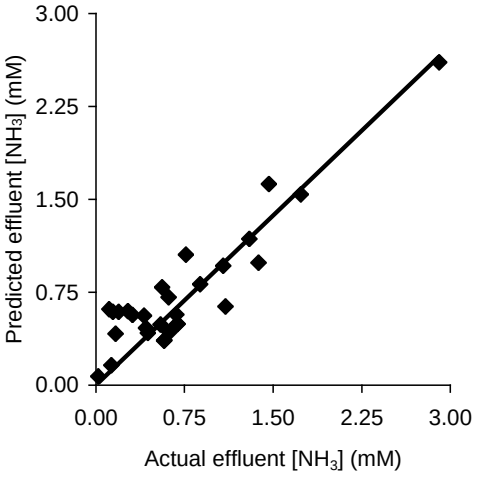

Figure E.15 Ammonia concentration in the effluent to the denitrification phase as measured compared to predicted. Slope = 0.9124, R^2 = 0.7770.

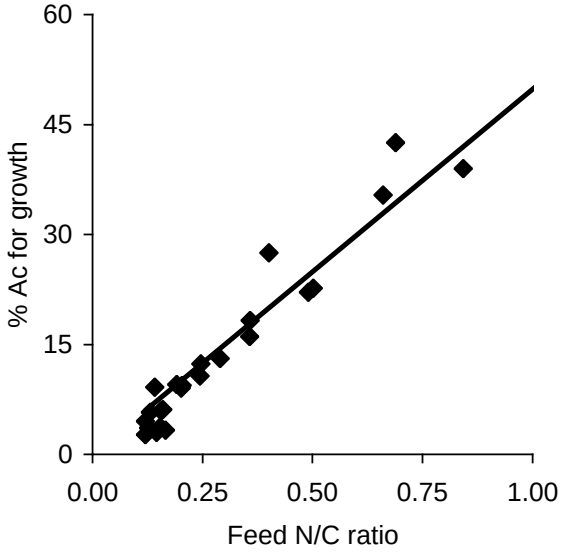

Figure E.16 Variation in estimated percentage of influent acetate used for biomass production with influent N/C ratio. Slope = 50.0, R^2 = 0.9212).

E.4.4 Comparison of assimilation estimation with mass balance results

The mass balance attempt with excess nitrate feed indicated that 13% of the influent carbon was used for growth in that experiment with a N/C ratio of 0.24 (Figures 2.10 and 2.11). This agrees very well with the prediction based on the analysis above (12%). According to Mara and Horan [132], an accepted overall equation including both catabolism and anabolism for denitrification is:

$$0.885\ CH_3COOH + 0.62\ HNO_3 + 0.2\ NH_3 \rightarrow 0.2\ C_5H_7NO_2 + 0.77\ CO_2 + 1.68\ H_2O + 0.305\ N_2$$

Equation E.14

This equation yields around 56% of influent carbon for assimilation. This relationship clearly does not take into account the changing conditions of the storage driven denitrification culture and the effect of varying N/C ratio on the relative predominance of growth and storage processes.

In the small scale mass balance experiments (Section 2.3.3), where 0.38 ± 0.05 moles of PHB were produced per mole of acetate taken up, this seems to contrast to the predictive relationship found above. In these experiments, the N/C ratio was about half that in the excess nitrate experiment and yet has resulted in the same storage outcome. If the context of the small scale experiments is considered, this becomes less problematic. The small scale experiments tested only the acetate uptake phase and since it is more likely that growth occurs in the denitrification phase by assimilation of stored substrate, the additional amount of storage observed would probably result in growth rather than denitrification if nitrate had been introduced.

The analysis of the accumulated data concerning the storage driven denitrification culture indicates, as expected, that the bulk of apparent ammonia metabolism (~89%) could be accounted for by a physical equilibration between the bulk liquor and the biofilm matrix in each phase. Only a small amount (~11%) was used for assimilation and this was most likely to occur during the denitrification phase. The actual values attributed to each process, adsorption or assimilation, should be treated with caution. However, for the purposes of modelling, this relationship provides a means to estimate the loss of ammonia through assimilation by the denitrification culture. The establishment of a bulk physical process for ammonia removal is a troubling result, as without substantial backwashing the storage driven denitrification biofilm and multistage reactors (see Chapter 4) will not be equipped for complete nitrogen removal. The subsequently developed PND reactor (Chapter 5) eliminates this issue.

Appendix F. Estimation of biomass in the biofilms

F.1 Biomass in the storage driven denitrification biofilm

The amount of biomass in the storage driven denitrification biofilm was estimated from the masses of biomass removed per bioball (approximately 0.1 g TS) during solid sampling throughout this study and the total number of bioballs (approximately 500) in the reactor. The total amount of biomass in the storage driven denitrification was estimated to be approximately 50 g, of which, 47% was volatile (Section 2.2.4).

F.2 Biomass in the nitrification trickling filter biofilm

Unlike the storage driven denitrification biofilm, there was little analysis required based on solid sampling so that a large amount of data could be collected to estimate the amount of biomass per bioball. Instead, the activity of the nitrification biofilm was compared to the activity of the nitrification SBR, with known biomass concentration.

The average rate of ammonia oxidation observed during SBR operation was 2.0 mM h^{-1} with a biomass (TS) concentration of 2.6 g L^{-1}.The average rate of ammonia oxidation observed during TF operation was 0.41 mM h^{-1}, which corresponds to approximately 0.53 g of biomass in the nitrification biofilm.

Appendix G. Estimation of oxygen free environment in the storage driven denitrification biofilm

Estimation of the oxygen free environment in the storage driven denitrification biofilm was required to eliminate aerobic denitrification activity to explain the consistent observation that PND could be performed at high soluble (> 2 mg L^{-1}) dissolved oxygen.

Parameter	Value
Average liquor flow rate	178 mL min^{-1}
Average liquor volume in the biofilm chamber	1250 mL
∴ Mean residence time of liquor	7 minutes
Average denitrification rate	1.25 mM h^{-1}
	0.021 mM min^{-1}
Estimate of concentration of DO in recycle vessel during high DO tests	4 mg L^{-1}
	0.125 mM (A)
Amount of oxygen consumed in mean liquor residence time (based on conservative denitrification rate)	0.147 mM (B)
Proportion of reactor during which consumption of oxygen would take place	A/B = 85%
ie proportion of reactor free of oxygen	**15%**

This estimate is likely to be much more conservative than in practice. The rate of consumption of oxygen is most likely to be higher than the rate of nitrate consumption by facultative heterotrophs. In addition, there is expected to be a high level of diffusion limitation through the biofilm that would contribute additional oxygen free environment within the biofilm.

Appendix H. Simple models of flow rate and type on nitrogen removal under PND operation

H.1 Effect of relative flow rate on nitrogen removal by PND

A simple spreadsheet model was constructed to probe the effect of relative flow rates in each column of the PND reactor. The kinetics of nitrification and denitrification were assumed to be first order due to substrate diffusion effects (Section 2.3.1). To simplify the model, several assumptions were made:

- The three vessels in the PND reactor (nitrification trickling filter, denitrification submerged biofilm and recycle vessel with no biological activity) were completely mixed flow reactors [62]. The major assumption in using completely mixed flow reactors is that any liquor entering the system is instantaneously mixed with the bulk liquor in that vessel;
- Flow rate through the recycle vessel is the sum of the flow rates through the nitrification and denitrification columns;
- No biological reaction occurs in the recycle vessel;
- Ammonia oxidation only occurs in the nitrification column and
- Nitrate reduction only occurs in the denitrification column.

Extreme data was chosen in order to accentuate any difference in behaviour with a different in relative flow rates.

Table H.1: Model input for recycle speed model.

Parameter	Nitrification column	Recycle vessel	Denitrification column
Vessel volume (L)	1.9	6.4	22
Feed [NH_3] (mM)	6	6	6
Feed [NO_3^-] (mM)	0	0	0
Flow rate (L/h) – "Fast nitrification"	11.99	13.21	1.22
Flow rate (L/h) – "Fast denitrification"	1.22	13.21	11.99
First order rate constant, k (h^{-1})	50	-	50

Model outcome indicates that the overall rate of ammonia removal is lower where the recycle speed through the denitrification column was significantly faster than through the nitrification column (Figure H.1).

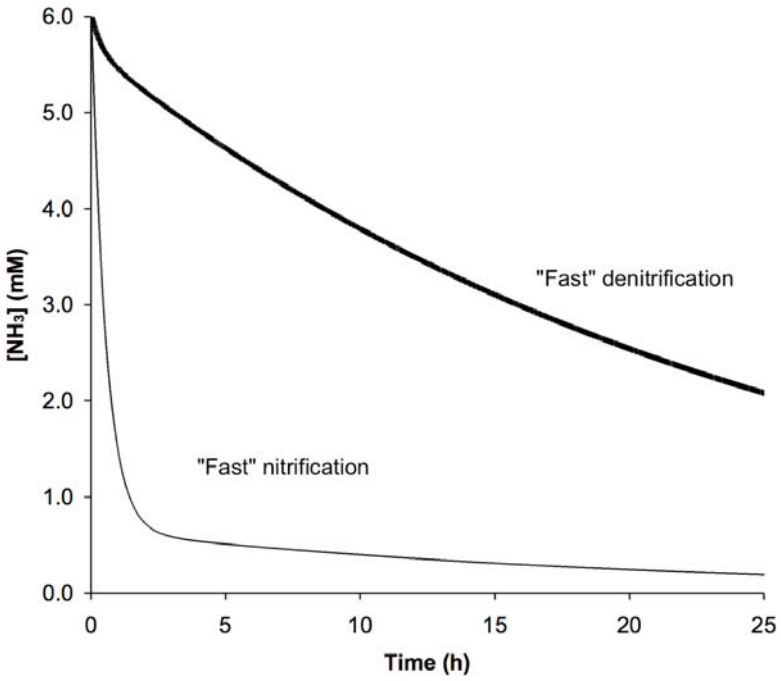

Figure H.1 Effect of relative recycle speed on the ammonia removal performance of the PND reactor.

H.2 Effect of direct or parallel flow on nitrogen removal by PND

A simple spreadsheet model was constructed to probe the effect of direct or indirect cycling of reactor liquor on the PND reactor. The kinetics of nitrification and denitrification were assumed to be first order due to substrate diffusion effects (Section 2.3.1). To simplify the model, several assumptions were made:

- The three vessels in the PND reactor (nitrification trickling filter, denitrification submerged biofilm and recycle vessel with no biological activity) were completely mixed flow reactors [62]. The major assumption in using completely mixed flow reactors is that any liquor entering the system is instantaneously mixed with the bulk liquor in that vessel;
- No biological reaction occurs in the recycle vessel;
- Ammonia oxidation only occurs in the nitrification column and
- Nitrate reduction only occurs in the denitrification column.

Extreme data was chosen in order to accentuate any difference in behaviour with a different in relative flow rates.

Table H.2: Model input for recycle style model.

Parameter	Nitrification column	Recycle vessel	Denitrification column
Vessel volume (L)	1.9	6.4	22
Feed [NH_3] (mM)	6	6	6
Feed [NO_3^-] (mM)	0	0	0
Flow rate (L/h)	30	30	30
First order rate constant, k (h^{-1})	50	-	50

Model outcome indicates that the overall rate of nitrogen removal is lower where the reactor liquor is cycled through each column independently rather than directly from one to the other (Figure 7.4). In addition, the transitional concentration of nitrate persisted longer where the reactor liquor is cycled through each column independently rather than directly from one to the other (Figure H.2).

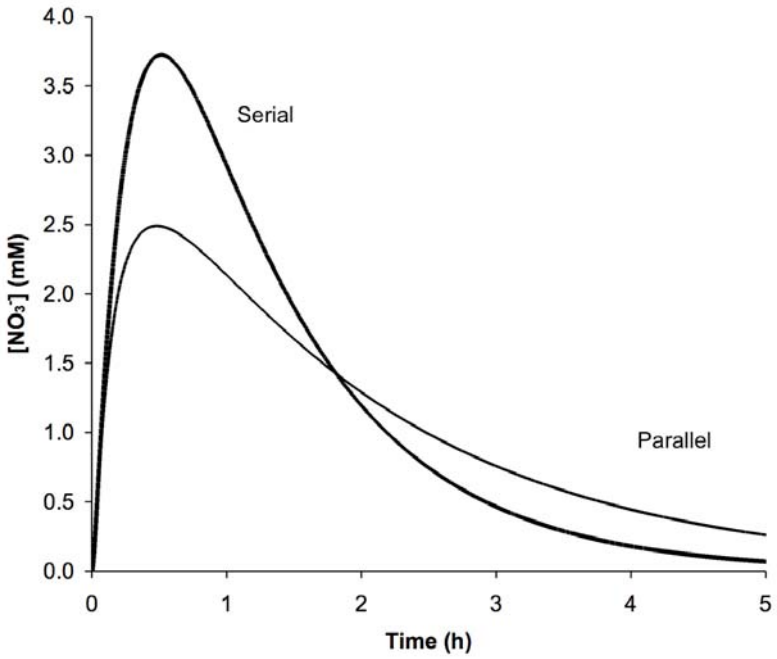

Figure H.2 Effect of recycle mode on the nitrate concentration in the recycle vessel of the PND reactor.